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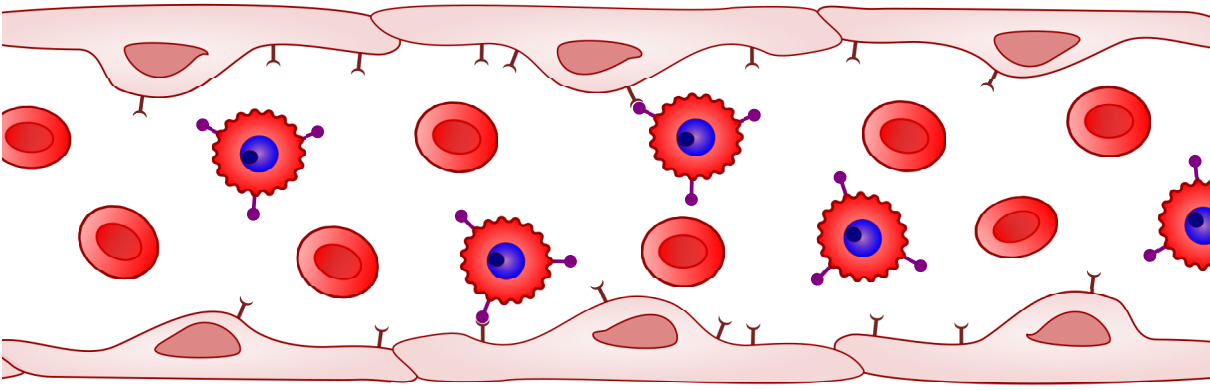
Malaria Parasites in Combination with Interferon-γ Induce Glucocorticoid Resistance in Pulmonary Endothelium



Karolina Zielińska

Promoter:
Prof. Philippe Van den Steen
Co-Promoters:
Prof. Karolien De Bosscher
Prof. Ghislain Opdenakker

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KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Rega Institute for Medical Research
Department of Microbiology and Immunology
Laboratory of Immunobiology

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MALARIA PARASITES IN COMBINATION WITH INTERFERON-Γ INDUCE GLUCOCORTICOID RESISTANCE IN PULMONARY ENDOTHELIUM

Karolina Zielińska

Dissertation presented in the partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

Jury:

Promoter: Prof. Philippe Van den Steen
Co-promoter: Prof. Karolien De Bosscher
Co-promoter: Prof. Ghislain Opdenakker
Chair: Prof. Marc Van Ranst
Secretary: Prof. Dirk Daelemans
Jury members: Prof. Jan Tuckermann, Ulm University, Germany
Prof. Geert Leroux-Roels, Ghent University, Belgium
Prof. Dominique Bullens, KU Leuven, Belgium
Prof. Dirk Daelemans, KU Leuven, Belgium

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Contents

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Summary

Malaria remains a global health problem causing more than 200 million clinical cases and 400 000 deaths each year. Athough most malaria infections remain uncomplicated, various complications, including cerebral malaria, severe malarial anemia, placental malaria and malaria-associated acute respiratory syndrome (MA-ARDS) cause the majority of deaths. MA-ARDS occurs mainly in adults and constitutes an important but insufficiently studied complication of malaria. MA-ARDS is characterised by lung edema, severe lung inflammation and impaired gas exchange. Currently, adequate treatment for this syndrome is not available. Therefore, our group developed an *in vivo* model of MA-ARDS with C57BL/6 mice infected with *Plasmodium berghei* NK65 (*Pb*NK65). In this model, extremely high doses of glucocorticoids (GCs) blocked development of MA-ARDS, but the highest doses acceptable in the clinic did not prevent the pathology. Furthermore, GCs failed to inhibit the expression of pro-inflammatory cytokines and chemokines in the lungs of infected mice. This suggests that malaria decreases GC sensitivity, but the mechanisms underlying GC resistance in malaria remain unclear.

This thesis aimed to identify molecular mechanisms of GC resistance in MA-ARDS. We established an *in vitro* model of GC resistance in malaria with lung endothelial cells. We investigated GC sensitivity of mouse microvascular lung endothelial cells stimulated with interferon-γ (IFN-γ) and *Pb*NK65. Upon challenge with IFN-γ alone, dexamethasone inhibited the expression of CCL5 (RANTES), CCL2 (MCP-1) and CXCL10 (IP-10). Accordingly, whole transcriptome analysis revealed that dexamethasone differentially affected several gene clusters and in particular inhibited a large cluster of IFN-γ-induced genes, including chemokines. This indicates that lung endothelial cells stimulated with IFN-γ remain GC sensitive. In contrast, combined stimulation with IFN-γ and *Pb*NK65 extract impaired inhibitory actions of GCs on chemokine

release reflecting GC resistance. However, the resistance did not affect the capacity of the GC receptor to accumulate in the nucleus. Subsequently, we studied the effects of GCs on STAT1 and MAPK pathways. Dexamethasone did not decrease phosphorylation and protein levels of STAT1. In contrast, dexamethasone inhibited the IFN-γ-induced activation of two MAPKs, JNK and p38. However, *Pb*NK65 extract abolished the inhibitory effects of GCs on MAPK signaling, inducing GC resistance. Overall, in this thesis we describe molecular mechanisms of regulation of IFN-γ-induced inflammation by GCs. We also identify *Pb*NK65 as a novel inducer of GC resistance in lung endothelial cells due to sustained MAPK activity. This work therefore provides novel insights and leads to the development of therapeutic strategies against MA-ARDS.

1 Introduction

1.1 Malaria remains a global health challenge

Malaria remains a life-threatening disease with negative impact on social and political stability. In 2015, 212 million clinical cases of malaria and approximately 429 000 deaths were reported (WHO website[1]). Malaria is caused by *Plasmodium* parasites, which belong to the *Apicomplexa* Phylum. Five species of *Plasmodium* can infect humans: *P. falciparum, P. vivax, P. knowlesi, P. malariae* and *P. ovale*. However, *P. falciparum* is responsible for the majority of deaths and infections. The *Plasmodium* parasite has a complicated life cycle. A female *Anopheles* mosquito injects sporozoites into the skin, which enter the blood stream and migrate into the liver. There, the parasites mature and undergo asexual replication. New merozoites released from hepatocytes enter the blood stream and infect red blood cells (RBCs). The parasite population grows exponentially in RBCs due to repeated cycles of invasion, replication and release. Some parasites differentiate into gametocytes within RBCs. These gametocytes can be taken up by a mosquito, in which sexual replication takes place resulting in a new infective mosquito. The first symptoms of malaria appear 7-15 days post infection during the erythrocytic stage and include fever, headache, muscle pain, chills and vomiting [1].

Most malaria infections remain uncomplicated. However, various complications including cerebral malaria, severe malarial anemia, placental malaria and malaria-associated acute respiratory distress syndrome (MA-ARDS) cause the majority of deaths [1, 2]. For example, cerebral malaria is a complication of *P. falciparum* infections that causes high mortality and post-recovery neurocognitive disorders. This complication is most common in sub-Saharan Africa. There, cerebral malaria is rare in adults and occurs mainly in children under

[1] http://www.who.int/malaria/en/

five years (WHO website[1]). Cerebral malaria also occurs in South East Asia, where malaria transmission is not intense enough to result in semi-immunity. In South East Asia, cerebral malaria mainly occurs in adults and older children [3]. Binding of infected RBCs (parasite sequestration) to the brain microvasculature plays a key role in the development of cerebral malaria [4]. In adults, cerebral malaria often occurs together with renal and respiratory failure. However, children suffer mainly from rapid onset of coma, anemia and seizures [5, 3].

1.2 MA-ARDS is a lethal and incompletely understood complication of malaria

MA-ARDS, occurs mainly in adults and constitutes an important but insufficiently studied complication of malaria [6, 7]. Patients with severe *falciparum, vivax,* and *knowlesi* malaria may suffer from MA-ARDS and this syndrome may even occur several days after anti-malarial treatment [7]. Most reported MA-ARDS cases arise in low-transmission areas or in non-immune travellers. Moreover, pregnant women with placental malaria may develop MA-ARDS. Currently, adequate treatment for MA-ARDS is not available [2, 8].

MA-ARDS is characterized by lung edema and impaired gas exchange [2]. Parasite-infected erythrocytes adhere to the endothelium in the lungs and this triggers leukocyte infiltration and pro-inflammatory cytokine production [2, 6]. Endothelial cells are activated by these cytokines and are likely the first cells altered in the lungs during acute respiratory distress syndrome (ARDS) [9]. Also, parasite products such as haemozoin and histidine-rich protein II have been shown to activate endothelium and increase endothelial barrier permeability [10, 11, 12]. Increased permeability of the microvascular barrier is typical in acute inflammation and plays a central role in the pathogenesis of ARDS

[1] http://www.who.int/malaria/en/

[9, 13, 8]. Moreover, it results in interstitial edema and facilitates leukocyte infiltration. Immune cells play a crucial role in the development of MA-ARDS. Abundant monocyte and macrophage infiltrates are found both inside the blood capillaries and in the interstitium in postmortem histological sections of patients with MA-ARDS [14]. Furthermore, lymphocytes and a small number of neutrophils are present [15]. In murine MA-ARDS, especially $CD8^+$ T cells are pathogenic [8]. Recent studies also suggested neutrophils to play a detrimental role, whereas monocytes appeared rather protective by phagocytosing infected erythrocytes [16, 17].

1.3 Sequestration and endothelial activation in malaria

Sequestration of infected RBCs in the microvessels allows the parasite to avoid clearance in the spleen. In mouse models of cerebral malaria, *P. berghei* ANKA-infected RBCs bind to murine brain and lung endothelial cells [18]. In humans, sequestration of *P. falciparum*-infected RBCs is mediated by interactions between members of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, encoded by *var* genes, and specific receptors on the endothelial cells. Switching between var genes enables immune evasion and affects the binding specificity of infected RBCs. Infected RBCs also form clusters with non-infected erythrocytes and this process is called rosetting [19].

Various receptors on the endothelial surface have been implicated in sequestration. Many parasite isolates bind to CD36, which plays an important role in innate immunity and phagocytosis of parasites during malaria. This has been associated with mild malaria [20, 21]. In human dermal endothelial cells, cytoadherence of infected RBCs to CD36 depends on Src family kinase activity. Treatment of human dermal endothelial cells with Src family kinase inhibitor significantly reduced adhesion of infected RBCs [22]. CD36 also mediates sequestration in adipose tissue and lungs in mouse models with *P. berghei* [23].

Two specific PfEMP1 subtypes containing domain cassettes (DCs) 8 and 13 are associated with severe childhood malaria. The interaction between DC8 or DC13 PfEMP1 and endothelial lining is mediated by endothelial protein C receptor (EPCR). EPCR is mainly involved in anti-inflammatory and cytoprotective effects of activated protein C [4].

Intercellular adhesion molecule 1 (ICAM-1) is another endothelial receptor that mediates sequestration in malaria. *P. falciparum* and *P. vivax* have been shown to bind to ICAM-1 [24, 25]. ICAM-1 also mediates sequestration in the *P. chabaudi* model [26]. Various cytokines enhance the expression of ICAM-1. In subcutaneous endothelial cells from patients with complicated malaria, tumor necrosis factor (TNF) induced higher expression of ICAM-1 compared to uncomplicated controls [27]. TNF also synergizes with interferon-γ (IFN-γ) to enhance ICAM-1 expression in mouse brain endothelial cells [28]. Other adhesion receptors such as platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are also involved in sequestration in malaria [29, 30].

Sequestration of infected RBCs disrupts blood flow, decreases endothelial barrier integrity and activates pro-inflammatory and coagulation pathways [31]. Ruptured *P. falciparum*-infected RBCs activate β-catenin and disrupt inter-endothelial junctions in human brain endothelial cells. Blockade of the angiotensin II type 1 receptor (AT1) or activation of the angiotensin II type 2 receptor (AT2) inhibited infected RBCs-induced activation of β-catenin and preserved endothelial barrier integrity [32]. *P. falciparum*-derived histones have also been shown to damage endothelial barrier [33]. The underlying mechanism involves impaired expression of junctional proteins and cell death. Stimulation of endothelial cells with *P. falciparum* histones also induces production of various pro-inflammatory mediators including interleukin (IL)-8, CC chemokine ligand (CCL)2, also known as monocyte chemoattractant protein 1 (MCP-1)

and cyclooxygenase 2 (COX2) via Src and p38 mitogen-activated protein kinase (MAPK) signaling pathways. Brain endothelial cells internalize and cross-present *P. berghei* ANKA antigens in the presence of IFN-γ and this confers susceptibility to killing by $CD8^+$ T cells leading to further disruption of the endothelial barrier [34].

The Tie receptors (Tie1 and Tie2) and their angiopoietin ligands (ANG1-ANG4) constitute a receptor Tyr kinase system in the vasculature. ANG-Tie controls vessel quiescence and regulates late stages of angiogenesis [35]. ANG1 exerts its anti-inflammatory effects through inhibition of vascular endothelial growth factor (VEGF)-induced expression of ICAM-1, VCAM-1 and E-selectin [36]. Moreover, ANG1 prevents VEGF and TNF-induced expression of the pro-coagulatory molecule tissue factor (TF) [37]. ANG2 was described as a ligand that antagonizes ANG1 activity on Tie2 and acts as a switch between the quiescent and inflamed state of endothelium [38, 39]. ANG2 also controls late steps of leukocyte adhesion. Furthermore, ANG2 sensitizes endothelial cells towards TNF and modulates TNF-induced expression of adhesion molecules [39]. Decreased ANG1 expression and soluble Tie-2 expression is associated with disease severity in patients with severe malaria. ANG1 also preserves endothelial barrier integrity in a mouse model of cerebral malaria [40]. Another study showed that in patients with malaria platelets release ANG1, possibly to mitigate harmful effects of ANG2 on endothelium [41].

1.4 Endothelial activation during inflammation

At rest endothelial cells present a nonreactive surface at the interface between blood and tissue. Upon activation the endothelium becomes a major player in the generation of the inflammatory response [42]. Pro-inflammatory cytokines produced by immune cells activate the endothelium and mediate leukocyte recruitment. Endothelial selectins such as E- and P-selectin capture leukocytes

from the blood flow and mediate rolling [43]. This slows down the circulating leukocytes and enables chemokines, localized on the endothelial surface, to interact with their receptors on leukocytes leading to integrin activation [44]. Pro-inflammatory mediators such as TNF, IL -1, -17A, -18, -33, -36γ and interferon (IFN) -α, -β, -γ stimulate the endothelium to produce various chemokines CCL2 (MCP-1), CCL20 (also known as macrophage inflammatory protein-3α (MIP-3α)), CXC chemokine ligand (CXCL)1 (also known as growth related oncogene-α (GRO-α)), CXCL8 (also known as IL-8), CXCL9 (also known as monokine induced by IFN-γ (MIG)), CXCL10 (also known as interferon-γ-induced protein 10 (IP-10)), CXCL11 (also known as interferon-inducible T cell α chemoattractant (I-TAC)) and also integrin ligands such as ICAM-1 and VCAM-1 [42, 45, 46, 47, 48, 49, 50, 51]. In contrast, IL-10 and IL-37 decrease CXCL8 (IL-8) and ICAM-1 expression in endothelial cells [52, 53]. ICAM-1 and VCAM-1 control firm adhesion and leukocyte crawling on the endothelial surface until they transmigrate through the endothelial barrier [54]. PECAM-1 controls localization of junctional proteins such as VE-cadherin and β-catenin and mediates leukocyte extravasation to the sites of inflammation [55].

1.5 Glucocorticoids are efficient anti-inflammatory molecules

1.5.1 Endogenous glucocorticoid synthesis and glucocorticoid receptor

Endogenous glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) are steroidal hormones synthesized from cholesterol in the adrenal cortex. Activation of hypothalamic-pituitary-adrenal (HPA) axis by stress, physical activity and trauma results in GCs production and release. The HPA axis is also activated by pro-inflammatory cytokines such as TNF. This results in GC secretion and inhibition of inflammation. GC activity is locally

regulated by extracellular binding proteins and intracellular enzymes. Only approximately 5% of circulating cortisol remains free and active. The rest binds to corticosteroid-binding globulin (CBG) [56].

Two distinct enzymes that regulate tissue specific metabolism of GCs exist. 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) converts inactive cortisone into cortisol. This isoform is expressed in liver, adipose tissue, brain, lungs, inflammatory cells, and gonads. In contrast, 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) inactivates GCs and allows aldosterone access to mineralocorticoids receptors in the classic aldosterone target tissues including distal nephrons, colonic epithelium and sweat and salivary glands [57, 58]. Moreover, two different receptors fine tune GC signaling: mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). High expression of MR has been reported in aldosterone target tissues such as kidney, colon, salivary glands, and specific brain regions. In contrast, GR is ubiquitously expressed [58]. MR binds with high-affinity both mineralocorticoids and GCs whereas GR is selective for GCs [59, 60]. Therefore, the occupancy of GR by GCs increases during stress and pharmacotherapy [58].

1.5.2 GR structure and isoforms

GR (NR3C1) mediates physiological and pharmacological effects of GCs. GR belongs to the nuclear receptor family of ligand-activated transcription factors. GR has a modular structure and is comprised of 3 domains (Figure 1): an N-terminal transactivation domain (NTD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) [61]. The NTD domain is a primary target for posttranslational modifications. Moreover, NTD contains activation function (AF)-1 which interacts with coregulators and the basal transcription machinery [62]. The DBD contains 2 zinc finger motifs that bind target DNA sequences – glucocorticoid response elements (GREs)

[61]. DBD and LBD are separated by a flexible region called hinge region (Figure 1). The LBD, which forms a hydrophobic pocket for binding GCs, also contains a second AF-2 domain. LBD and the DBD-hinge region contains nuclear localization signal (NLS)1 and NLS2 [62].

The human GR gene contains 9 exons. Alternative splicing in exon 9 generates two GR isoforms: GRα and GRβ (Figure 1). GRα is the classic GR protein that mediates GC actions. GRβ resides constitutively in the nucleus and does not bind ligands. GRβ is expressed in many tissues and cell types but generally at lower levels than GRα and has been shown to inhibit GRα. GRβ competes with GRα for GRE binding and transcriptional coregulators. Furthermore, GRβ forms inactive dimers with GRα [62]. GRγ, GR-A, and GR-P are additional GR splice isoforms [61]. The expression of GRγ correlates with GC resistance in childhood acute lymphoblastic leukemia [63]. GR-A is not well studied whereas GR-P is expressed in GC-resistant cancer cells [62].

Alternative translation initiation generates eight GRα isoforms with progressively shorter N-terminus: GRα-A, -B, -C1, -C2, -C3, -D1, -D2, and -D3, adding another level of complexity to the GR biology. Other GR splice variants would also generate various translational isoforms. GRα isoforms exhibit a similar ligand binding affinity and similar GRE binding capacity. However, their cellular localization differs [61]. Human GRα-D (hGRα-D) constitutively resides in the nucleus. In contrast, hGRα-A, -B and C localize in the cytoplasm and translocate to the nucleus upon ligand binding. Furthermore, each GRα isoform regulates a unique and common set of genes [64]. All isoforms GR are also subject to a variety of posttranslational modifications including phosphorylation, ubiquitination and SUMOylation (Figure 1) [62].

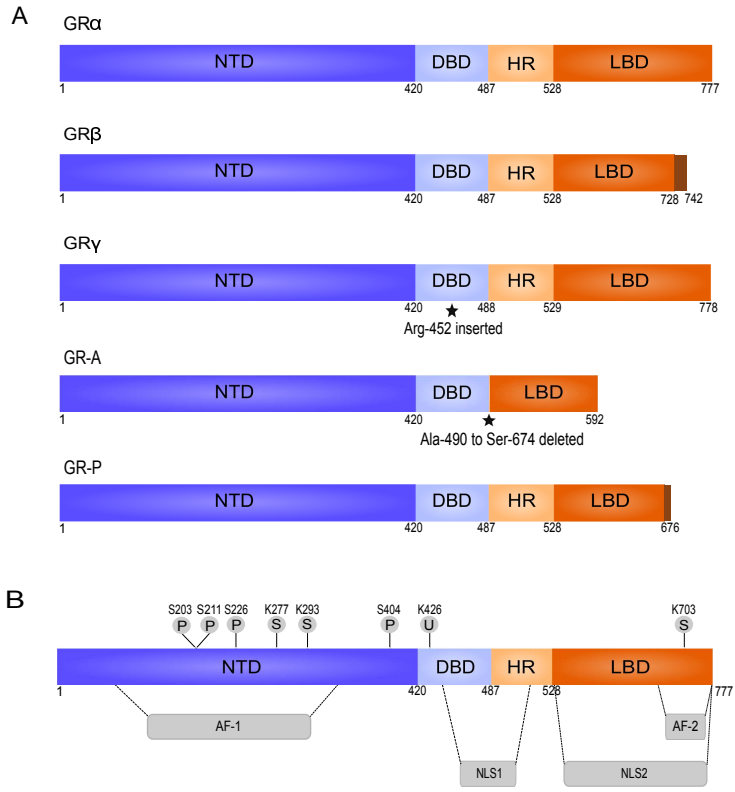


Figure 1: Domain structure of the human GR (A) with its target residues for posttranslational modifications (B). AF – activation function, DBD – DNA-binding domain, HR – hinge region, LBD – ligand binding domain, NLS – nuclear localization signal, NTD – N-terminal domain, P – phosphorylation, S – sumoylation, U – ubiquitination.

1.5.3 Molecular mechanism of GC action

In the absence of ligand GR resides mainly in the cytoplasm in complex with several proteins: Hsp90, Hsp70, p23, FKBP51 and FKBP52 [65]. Upon ligand binding GR dissociates from the multiprotein complex and translocates into the nucleus. Inside the nucleus GR (as a homodimer) binds to the consensus GRE sequence GGAACAnnnTGTTCT, which is an imperfect palindrome [62].

Upon GRE binding GR undergoes conformational changes leading to coregulator and chromatin-remodeling complexes recruitment. This process influences the activity of RNA polymerase II [61]. The consensus GRE sequence, often referred to as positive GRE, has been shown to mediate induction of various GR-dependent genes including sphingosine kinase 1 (SphK1) and TGF-stimulated clone 22 domain protein-3 (Tsc22d3), also known as glucocorticoid-induced leucine zipper (GILZ) [66, 67]. However, only a small subset of GREs are occupied by GR. The specific sites where GR binds vary among tissues and depend on chromatin accessibility [68]. Activator protein 1 (AP-1) plays an important role in the maintenance of baseline chromatin accessibility and facilitates GR recruitment [69].

Negative GREs (nGREs), which mediate GR-dependent transcriptional inhibition, have been also identified in the genome [70]. In contrast to the positive GRE sequence, the consensus nGRE sequence CTCC(n)0-2GGAGA, which contains a variable spacer that ranges from 0 to 2 nucleotides, can be occupied by 2 GR monomers that do not dimerize [62]. Hudson and colleagues showed that in the nGREs, each GR monomer is oriented away from the second one preventing interaction between dimerization loops [71]. Ligand bound GR might also influence gene expression via protein-protein interactions with other transcription factors (TFs) bound to DNA. For example, the GR signal transducer and activator of transcription (STAT)5 complex activates gene transcription [72, 73]. In contrast, GR recruitment to DNA bound transcription factors such as AP-1 and nuclear factor κB (NF-κB) inhibits their activity. This is a primary mechanism by which GCs inhibit inflammation. GR interferes with the transactivating capacity of p65 and c-Jun [62]. Several nonexclusive mechanisms of NF-κB inhibition by GR have been reported including GR-mediated tethering of the NF-κB p65 subunit, histone deacetylase 2 (HDAC2) recruitment by GR to the NF-κB-dependent promoters, and blockade of transcriptional elongation

[74]. Moreover, GR activation blocks NF-κB interaction with chromatin [75]. AP-1, which can be tethered by GR, also regulates GR binding to DNA and facilitates productive GR-chromatin interactions [69]. Furthermore, GR can also inhibit gene transcription in a composite manner via binding to GRE and physical association with AP-1 bound to DNA [76].

1.5.4 Non-genomic actions of GR

GR can also exert some of its actions via non genomic mechanisms that do not require protein synthesis and occur within a few seconds to minutes [62]. Nongenomic effects of GCs are mediated through cytosolic and membrane bound GR [61]. Dexamethasone blocks arachidonic acid release in A549 cells via inhibition of recruitment of signaling factors to the epidermal growth factor receptor. This inhibition is GR-dependent but transcription-independent [77]. GCs induce rapid serine phosphorylation and membrane translocation of ANXA1 via nongenomic pathways in a human folliculostellate cell line resulting in inhibition of adrenocorticotropic hormone (ACTH) secretion [78]. In thymocytes, GR translocates to mitochondria and induces apoptosis or affects mitochondrial gene expression [79].

1.5.5 Effects of GCs on immune cells

The effects of GCs on immune cells have extensively been studied. For a complete overview of the literature on this subject the reader is referred to extensive reviews [56, 80]. Here, several examples of interesting effects of GCs on specific leukocytes populations are described. In B cells, GCs induce cytotoxic and growth-suppressive effects via induction of GILZ. GILZ KO mice develop B lymphocytosis due to increased B cell survival. Moreover, impaired B cells apoptosis in those mice correlates with enhanced NF-κB activity and Bcl-2 expression [81]. In contrast, GCs have been shown to inhibit apoptosis

in neutrophils [82]. Dexamethasone induces dichotomic effects, dependent on the cytokine milieu, in NK cells. It initially suppresses IFN-γ production but enhances proliferation and survival in NK cells challenged with IL-2 and IL-12. Furthermore, NK cells restimulated with IL-2, IL-12 and dexamethasone showed enhanced IFN-γ production [83].

GCs regulate multiple aspects of T cell physiology. For example, in patients with multiple sclerosis (MS), high-dose GCs induce apoptosis in $CD4^+$T cells [84]. Moreover, GCs inhibit IL-2 release and T cell receptor (TCR) signaling as well as phosphorylation of several molecules down-stream of TCR including p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), protein kinase (PK)B and PKC [85, 86]. GCs have also been shown to induce regulatory T cells that express the transcription factor forkhead box P3 (FoxP3). The underlying mechanism is induction of GILZ by GCs. GILZ enhances transforming growth factor β (TGF-β) signaling and activates FoxP3 expression. *In vivo*, GILZ overexpression increases T reg cell number, whereas GILZ KO impairs generation of peripheral regulatory T cells [87]. Interestingly, Th2 cells produce a steroid pregnenolone that inhibits Th cell proliferation and B cell class switching. This *de novo* pregnenolone synthesis might represent a unique way of Th2 responses to restore immune homeostasis [88].

GCs impair dendritic cells (DCs) differentiation and induce a population of DCs incapable of inducing effective immune response. These DCs fail to prime Th1 cells [89]. GC administration during DC maturation reduces IL-12 p70, TNF production and T cell stimulatory function. In patients with systemic autoimmunity, GCs decrease the number of myeloid DCs and abrogated plasmacytoid DCs. Although GCs enhanced toll-like receptor (TLR)2, 3, and 4 expression on DCs, challenge with TLR ligands failed to induce maturation [90]. However, the effects of GCs on DCs depend on the maturation state since the inhibitory effect of GCs on DCs was absent upon challenge with

lipopolysaccharide (LPS) [89]. GCs have also been shown to induce apoptosis in human plasmacytoid pre-dendritic cells [91].

The effects of GCs on macrophages are concentration-dependent. Low concentrations of GCs enhance pro-inflammatory cytokine and nitric oxide (NO) expression. In contrast, high doses of GCs suppress macrophage function [92]. These data are in contrast with another study from Van de Garde and colleagues who compared the transcriptome of human macrophages matured in the presence or absence of high-dose GCs and the ability to initiate or maintain classic activation [93]. Macrophages were challenged with acute LPS and chronic IFN-γ stimulation. Long-term stimulation with GCs weakens adaptive immune signature components of IFN-γ and LPS gene profile via down-regulation of major histocompatibility complex (MHC) class II, but strengthens innate signature components through induction of chemokines that attract leukocytes. In general, high doses GCs do not suppress macrophage function, but shift the balance between innate and adaptive immunity. GCs have also been shown to inhibit p38 pathways in macrophages challenged with LPS [94]. In monocytes, GCs induce anti-inflammatory genes such as GILZ and IL-10 leading to M2 polarity [95].

1.5.6 Effects of GCs on endothelial cells

It becomes more and more clear that GCs also regulate multiple aspects of endothelial physiology (Figure 2). GCs inhibit pro-inflammatory signaling pathways in endothelium and induce protective molecules that maintain endothelial function, in particular upon inflammation. For instance, dexamethasone blocks nuclear translocation of NF-κB and reduces the binding of AP-1 and GATA to DNA in endothelial cells [96]. GCs induce map kinase phosphatase-1 (MKP-1), also known as dual-specificity phosphatase-1 (DUSP1), which inhibits MAPK signaling pathways, and tristetraprolin (TTP), also known as

(ZFP36), which destabilizes mRNAs of pro-inflammatory cytokines [97, 98]. Annexin A1 (AnxA1), induced by GCs in endothelium, causes leukocyte detachment and regulates BBB integrity [99, 100, 101]. Moreover, AnxA1 inhibits phospholipase A2, an enzyme that releases arachidonic acid from phospholipids to produce pro-inflammatory mediators such as prostaglandins and leukotrienes via cyclooxygenase [102].

GCs inhibit the endothelial production of several pro-inflammatory cytokines and chemokines such as IL-6, IL-17F, CXCL8 (IL-8) and CCL2 (MCP-1) [98, 97, 103]. GCs also down-regulate ICAM-1, VCAM-1 and E-selectin [104, 105, 106]. Moreover, GCs reduce the levels of soluble forms of ICAM-1, VCAM-1 and E-selectin (sICAM-1, sVCAM-1, sE-selectin) [107]. Interestingly, GCs may also down-regulate HLA-DR in IFN-γ-stimulated endothelial cells [104]. Overall, GCs decrease leukocyte transmigration across the endothelium, thus limiting inflammation [108].

GCs also increase the activity of eNOS – a critical mediator of vascular integrity [109]. Release of NO in the lumen inhibits platelet aggregation and leukocyte adhesion [110]. Conversely, Iuchi et al. showed that GC excess induces reactive oxygen species and peroxynitrite formation, with possible detrimental effects on the vasculature [111].

Disruption of the endothelial barrier integrity is a common feature of various diseases including MS and stroke and leads to edema [112]. GCs preserve endothelial barrier integrity through up-regulation of junctional proteins such as occludin, claudin-5 and VE-cadherin [113, 114, 115] and down-regulation of matrix metallopeptidase 9 (MMP-9) – an enzyme involved in junctional protein cleavage [116, 117, 118]. GCs also inhibit MMP-9 via induction of tissue inhibitor of metalloproteinases (TIMP) – TIMP-3 and TIMP-1 [119, 117]. However, the induction of TIMP-1 seems controversial, since contradictory results exist [119]. Since MMP-9 is able to cleave CXCL8 (IL-8) and drastically po-

tentiates its activities [120], it is tempting to speculate that down-regulation of MMP-9 by GCs further affects neutrophil chemoattraction at the endothelial surface.

In summary, GCs influence endothelial barrier integrity, inhibit various pro-inflammatory transcription factors, and induce protective molecules in endothelium (Figure 2). Investigation of molecular mechanisms of GC action in endothelium will enable comparison of these to the extensive data on regulation of inflammation by GC in other cell types.

1.5.7 GCs in the clinic

GCs have been prescribed as therapy for various inflammatory diseases including rheumatoid arthritis, asthma and multiple sclerosis since the 1950s [56, 121]. Synthetic GCs are more potent immunoregulators than endogenous ones because they are not targets of endogenous inhibitors such as 11β-HSD and CBG. Furthermore, synthetic GCs bind the GR with higher affinity and the MR with lower affinity than endogenous hormones [56]. However, GC therapy has two major drawbacks: side-effects and GCs resistance.

Although GCs have been an effective therapy in chronic inflammatory diseases, the long term therapy with GCs results in severe side-effects including diabetes, bone frailty, muscle wasting, fat depot changes, hypertension, susceptibility to infections, and mood disorders [122]. Different GR conformations reflect the complex nature of GR and exert diverse anti-inflammatory profiles. GR agonists currently used in the clinic might activate multiple anti-inflammatory actions of GR. Such an excessive non-specific anti-inflammatory response results in side-effects and limits the beneficial effects of the therapy. Since the biology of the GR is complex, selective GR-mediated activation of anti-inflammatory profiles provides a means to develop disease-tailored therapies. For example, treatment that induces formation of GR monomers might

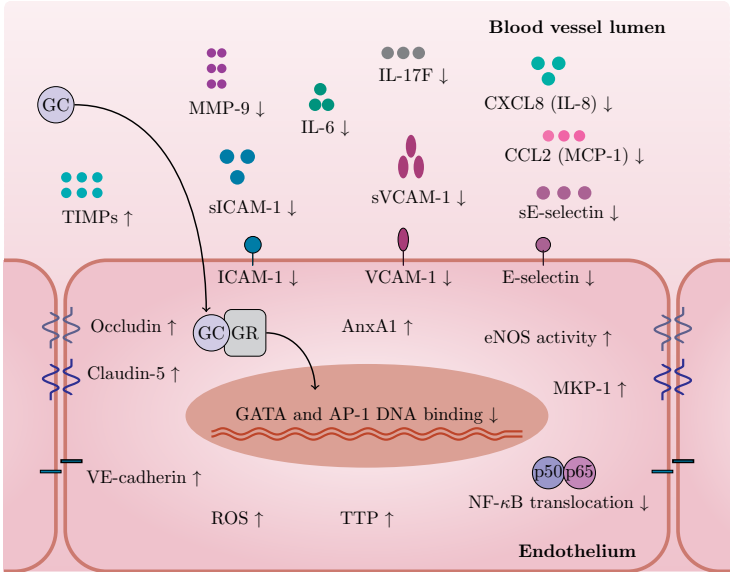


Figure 2: GCs exert specific actions in endothelial cells. In general, GCs have a variety of cell-type specific effects. This figure depicts GC actions that have been described in endothelium. After entering an endothelial cell, GCs bind to GR and translocate to the nucleus. GR bound to GCs inhibits pro-inflammatory pathways by limiting GATA and AP-1 DNA binding and NF-κB translocation. GCs decrease levels of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and also their soluble forms and MMP-9 while increasing levels of junctional proteins – occludin, claudin-5 and VE-cadherin. GCs induce protective molecules such as AnxA1, TTP, MKP-1 and TIMPs. Furthermore, GCs reduce levels of IL-6, IL-17F, CXCL8 (IL-8) and CCL2 (MCP-1). Stimulation with GCs increases the activity of eNOS – a critical mediator of vascular integrity. Induction of ROS represents detrimental effects of GC excess on the vasculature.

help to reduce the number of side effects during GC therapy in chronic inflammatory diseases. In contrast, patients with acute inflammation would benefit from skewing the GR phenotype towards dimers [121]. Animal models suggest that GR-induced anti-inflammatory genes such as SphK1 confer protection against acute inflammation [67].

GC resistance has been reported in various diseases such as asthma and

chronic obstructive pulmonary disease (COPD) [123] and in different cells types including peripheral blood mononuclear cells (PBMCs), B cells and alveolar macrophages. A wide range of mechanisms causing GC resistance in these cell types have been described, including increased GRβ expression, post-translational GR modification, impaired nuclear translocation of GR, reduced MKP-1 (DUSP1) expression and decreased activity of HDAC2 [124, 125, 126, 127, 128]. Furthermore, deregulated microRNA expression might contribute to GC resistance. For example, microRNA-124 has been shown to target GR and reduce its levels in acute lymphoblastic leukemia (ALL) [129]. Also in ALL, NLRP3-CASP1 inflammasome might cleave GR leading to reduced GR-mediated transcriptional response and GC resistance [130].

1.6 Regulation of endothelial GC sensitivity

A limited number of studies addressed GC sensitivity of endothelium and uncovered several mechanisms that regulate the response of endothelium to GCs (Figure 3). In dexamethasone-resistant human umbilical vein endothelial cells (HUVECs), GR interacted more strongly with BCL2-associated athanogene 1 (BAG1) protein than in dexamethasone-sensitive HUVECs [131]. In general, BAG1 is a cytoplasmic protein, which can translocate to the nucleus and bind to DNA, decreasing GR transactivation [132]. BAG1 also interferes with GR folding through interaction with Hsp70 [133]. Moreover, BAG1 may target interacting proteins for proteasomal degradation [134]. In HUVECs, proteasome inhibition increased GR protein levels and abolished differences between GC-sensitive and GC-resistant cells, suggesting that BAG-1 mediated proteasomal degradation of GR accounts in part for the human variability in endothelial sensitivity to GCs [131]. *In vivo*, proteasome inhibition improved GC sensitivity of endothelium and protected against brain edema [135].

Epigenetic mechanisms such as DNA methylation and histone modifica-

tions also regulate GC sensitivity of endothelial cells. Dexamethasone-sensitive and dexamethasone-resistant HUVECs have different GR promoter methylation patterns [136]. The GR gene contains several variants of the untranslated exon 1: 1A–1I [137]. Each of these variants has its own promoter. Dexamethasone-sensitive cells show higher methylation levels of promoter 1D and lower methylation levels of promoter 1F. Pharmacological demethylation with 5-aza-2-deoxycytidine increased the mRNA expression of all isoforms (except 1D in resistant HUVECs) and enhanced the GC sensitivity [136]. Another epigenetic mechanism that influences endothelial GC sensitivity involves Sin3A-HDAC. This multi-protein complex that regulates gene expression via histone deacetylation consists of SAP30, Sin3, histone deacetylases HDAC1 and HDAC2, histone binding proteins RbAp46 and RbAp48, and other proteins. SAP30 represses gene transcription via tethering to gene promoters [138]. Poor expression of Sap30 has been suggested as an explanation of impaired transrepression in HUVECs [139]. Transgenic overexpression of SAP30 in HUVECs and analysis of its expression under inflammatory conditions would also improve the current understanding of how GC sensitivity is regulated in endothelium.

Variations in the expression of the GC-activating and -deactivating enzymes 11β-HSD1 and 11β-HSD2 have been described in endothelial cells [140]. However, it remains unclear to what extent this affects the GC sensitivity.

In conclusion, the currently known mechanisms regulating GC sensitivity in endothelial cells summarized in Figure 3 include proteasomal degradation of GR and epigenetic modifications. However, the mechanisms that control GC sensitivity are complex and often cell-type specific. The regulation of GC sensitivity in endothelial cells has not yet been sufficiently addressed, in particular under inflammatory conditions. Since endothelium plays a crucial role in inflammation, we believe that further studies on this subject will open up new

perspectives for the development and improvement of the current treatment strategies.

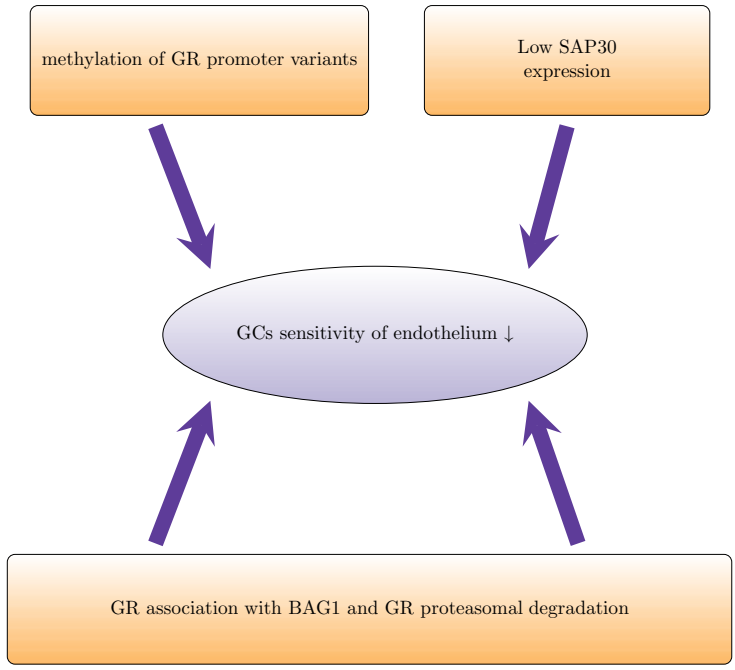


Figure 3: Proteasomal degradation and epigenetic modifications regulate GC sensitivity in endothelial cells. Proteasomal degradation of GR impairs GR activity and prevents physiological actions of GCs. In dexamethasone-resistant HUVECs, GR associates with the proteasomal recruiting protein BCL2-associated athanogene 1 (BAG1). This results in a shorter half-life of GR. In HUVECs low induction of SAP30 (component of Sin3A-histone deacetylase complex) impairs GR-mediated transrepression and leads to lower GC sensitivity. Furthermore, higher methylation of the promoters of the different variants of the untranslated exon 1 of GR leads to lower expression and therefore decreased GC sensitivity.

1.7 Specific aims

Previously, our group developed a mouse model of MA-ARDS with C57BL/6 mice infected with *P. berghei* NK65. These mice develop MA-ARDS with increased vascular permeability, protein-rich lung edema and leukocyte infiltration. According to the observed histopathology, this mouse model exhibits important similarities to human MA-ARDS and is suitable to investigate both pathogenesis and therapeutic strategies [2]. In this model, extremely high doses of GCs (80 mg/kg) blocked development of MA-ARDS but failed to inhibit the expression of pro-inflammatory molecules in the lungs of infected mice. The highest doses acceptable in the clinic (3 mg/kg) did not prevent the pathology and failed to decrease the pulmonary expression of cytokines and chemokines. This suggests that malaria decreases GC sensitivity [8]. However, the mechanisms underlying GC resistance in MA-ARDS remain unknown. The general aim of this thesis was to identify molecular mechanisms of GC resistance in MA-ARDS. To this end, 3 specific steps were taken:

1. to establish an *in vitro* model of malaria induced GC resistance. Since this PhD project aimed to investigate the molecular mechanisms of malaria-induced GC resistance, we searched for a simple model with one cell type. In view of the importance of endothelium in malaria, I stimulated mouse lung endothelial cells with IFN-γ and *P. berghei* NK65 and investigated their GC sensitivity.

2. to study the GR signaling in mouse lung endothelial cells challenged with IFN-γ and malaria parasites in the presence or absence of GCs. Impaired GR signaling has been reported as one of the mechanisms underlying GCs resistance in various diseases. Therefore, I first investigated GR phosphorylation and nuclear translocation.

3. to evaluate the effects of GCs on STAT1 and MAPK signaling in mouse

lung endothelial cells challenged with IFN-γ and malaria parasites. IFN-γ activates two pro-inflammatory signaling pathways, STAT1 and MAPK. GCs have been shown to regulate both pathways in various experimental models. As a next step, I studied the effects of GCs on STAT1. Moreover, I also investigated the effects of specific MAPK inhibitors on the induction of inflammatory chemokines and the effects of GCs on MAPK signaling.

2 Development of an *in vitro* model of malaria-induced GC resistance

2.1 Introduction

Chemokines and cytokines regulate leukocyte migration during malaria and contribute to the pathogenesis of severe malaria. Increased levels of CXCL8 (IL-8) and CXCL9 (MIG) have been observed in patients with severe malaria. Furthermore, CXCL10 (IP-10) has been used as a biomarker for *P.falciparum*-induced cerebral malaria [141, 142]. In contrast, low levels of CCL5 (also known as regulated on activation, normal T cell expressed and secreted (RANTES)) in children with cerebral malaria correlate with mortality [143]. In the mouse model of cerebral malaria, upregulation of several chemokines, such as CXCR3 ligands have also been observed. CXCL9 (MIG) and CXCL10 (IP-10) were mainly expressed in cerebral microvessels and in adjacent glial cells, whereas CCL5 (RANTES) was predominantly produced by infiltrating lymphocytes. CXCR3 KO mice were protected from cerebral malaria and showed less $CD8^+$T cells in the brain [144]. CXCL10 (IP-10) deficiency has been shown to protect against cerebral malaria and to reduce parasite biomass *in vivo* [142].

IFN-γ plays an important role in controlling both the liver and the blood stage of malaria [145, 146, 147]. However, it can also aggravate malaria infections. For example, IFN-γ has been shown to exacerbate the pathology in animal models of cerebral malaria [145, 148]. IFN-γ synergizes with lymphotoxin-α and TNF to induce the expression of adhesion molecules (E-selectin and ICAM-1) [28]. Moreover, IFN-γ produced by $CD4^+$T cells upregulates CXCL9 (MIG) and CXCL10 (IP-10) in endothelial cells and induces $CD8^+$T cell recruitment into the brain [149, 150]. IFN-γ KO mice are protected from cerebral malaria and show lower leukocyte infiltration in the brain [151]. Similarly, in the IFN-γR1 KO mice infected with *Pb*ANKA leukocyte infiltration into the brain was

absent. However, leukocyte infiltration into the lungs was still observed in the KO mice. WT and KO mice also showed specific patterns of chemokine and chemokine receptor expression. Induction of CCL5 (RANTES), CXCL10 (IP-10) and CCR2 was associated with leukocyte infiltration into the brain, whereas upregulation of CCL2 (MCP-1), CXCL10 (IP-10) and CCR5 was associated with leukocyte migration into the lungs [152]. Interestingly, CXCR3 KO mice produce less IFN-γ and lymphotoxin-α in the brain [144].

In this chapter, I stimulated mouse lung endothelial cells (L2 MVECs) with IFN-γ and IFN-γ in combination with malaria parasites and evaluated their GC sensitivity. As a readout for GC sensitivity, I analyzed the effects of GCs on the induction of several chemokines such as CXCL10 (IP-10), CCL5 (RANTES) and CCL2 (MCP-1), which play an important role in malaria.

2.2 Materials and methods

2.2.1 Cell cultures

The murine lung microvascular endothelial cell line (L2 MVEC, a kind gift from Dr J. Brian De Souza, London School of Hygiene and Tropical Medicine) was cultured in RPMI medium (Gibco, Belgium) supplemented with 2 mM L-glutamine (Gibco), 0.1 mg/mL streptomycin (Sigma, Belgium), 200 U/mL penicillin (Kela, Belgium) and 10% FCS (Gibco). Cells were grown in 5% CO_2 at 37°C.

2.2.2 Mice

All animal experiments were performed in accordance to the regulations as declared in Directive 2010/63/EU from the European Union and and the Belgian Royal Decree of 29 May 2013 and were approved by the Animal Ethics Committee from the KU Leuven (project number P163-2014, License LA1210186, Belgium). All efforts were made to minimize suffering of animals. Unless oth-

erwise indicated, male Balb/c mice were obtained from Janvier (seven to eight weeks old, Le Genest-Saint-Isle, France). Mice were injected intraperitoneally with 10^6 *Pb*NK65-infected RBCs (a kind gift of the late Prof. D. Walliker, University of Edinburgh). Mice were kept in a conventional animal house and drinking water was supplemented with 4-amino benzoic acid (0.375 mg/ml, PABA, Sigma-Aldrich, Bornem, Belgium). Parasitemia was determined by microscopic analysis of blood smears of tail blood after Giemsa staining (1/10 dilution, VWR, Heverlee, Belgium). Mice were sacrificed 8 days after infection (when parasitemia was approximately 4%) by euthanasia with Dolethal (Vtoquinol, Aartselaar, Belgium; 200 mg/ml, intraperitoneal injection of 50 μl) and cardiac punctures were performed.

2.2.3 *Ex vivo* cultivation of *Pb*NK65 and extract production

After cardiac punctures blood was filtered with Plasmodipur filters (Europroxima) to remove leukocytes and washed with RPMI medium (Gibco) supplemented with: 25 mM HEPES, 0.425 g $NaHCO_3$, 2 mM L-glutamine (Gibco), 5 mM glucose (Sigma) and 20% FCS. The pellet was resuspended and seeded into culture flasks. Cells were gassed with a mixture of 92.5% N_2, 5.5% CO_2, 2% O_2 and cultured overnight at 37°C. The next day the culture was centrifuged, resuspended in RPMI medium and loaded on MACS column (Miltenyi Biotec, NL) to purify the schizonts on the basis of their inherent magnetic properties. After elution the schizonts were diluted to the concentration of 10^8/mL in RPMI medium, aliquoted and frozen at -20°C. Next they were thawed and frozen at -20°C again to produce the extract.

2.2.4 Stimulation of L2 MVECs with IFN-γ, *Pb*NK65 extract and cytokine determination

L2 MVECs were seeded in 6, 24, 48 or 96 well plates at the concentration of $5{\cdot}10^4$ cells/mL. After expanding the cells for 24 hours, they were washed with

medium and stimulated for 24 hours with combinations of murine IFN-γ (20 ng/mL, PeproTech, USA), parasite extract (10^7 infected RBCs – iRBCs/mL), murine RBCs extract (10^7 RBCs/mL), and dexamethasone (100 nM final concentration, diluted form a stock solution of 0.06M dissolved in DMSO, Sigma). After stimulation plates were centrifuged (5 minutes, 1200 rpm, RT) and supernatants were collected for ELISA and stored at -20°C. Cytokines were analyzed by ELISA (R&D).

2.2.5 Quantitative RT-PCR and RNA-seq

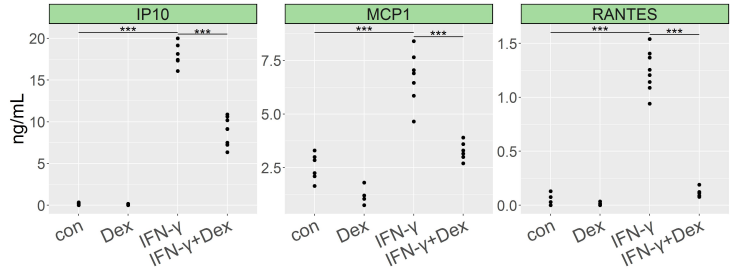
Cells were washed with PBS, lysed using RLT buffer with β-mercaptoethanol from the RNeasy Mini kit (Qiagen, Belgium) and stored at -80°C for RNA extraction. RNA was extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were evaluated with Nanodrop 1000 (Thermo Scientific, Belgium). RNA (0.25μg) was converted to cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR was performed with 6.25 or 0.125 ng cDNA using predesigned primers (IDT) and TaqMan Universal PCR Master Mix (Applied Biosystems), respectively. RNA-Seq expression profiling was performed by the Genomics Core UZ Leuven. Per independent experiment RNA from 3 technical replicates per experimental condition was pooled and 3μg RNA/experimental condition were sequenced from a total of 3 independent experiments. Illumina TruSeq stranded mRNA kit was used and the single-end sequencing was performed. 33M 50 bp reads per sample were sequenced. Reads were aligned to mm10 murine genome using TopHat. A heatmap with highly variable genes across the samples was plotted using pheatmap package (pheatmap). The rlog transformed counts of each gene were centered across the samples. Ribosomal RNA genes and predicted genes that were increased in one sample (GC027190) were excluded from the rlog data used for heatmap since this increase was

a result of an imperfect poly-A selection during the library preparation of this sample. Differential expression analysis was performed with DESeq2 [153]. Differences in gene expression with a FDR adjusted p value below 0.1 were considered significant. Gene ontology analysis of differentially expressed genes was performed with clusterProfiler package [154]. Motif analysis of the proximal promoter region (400bp upstream of the transcription start site till 100 bp downstream) was performed using Homer software (Homer motif analysis). RNA-seq data were submitted to ArrayExpress (accession number E-MTAB-5921, (ArrayExpress URL)).

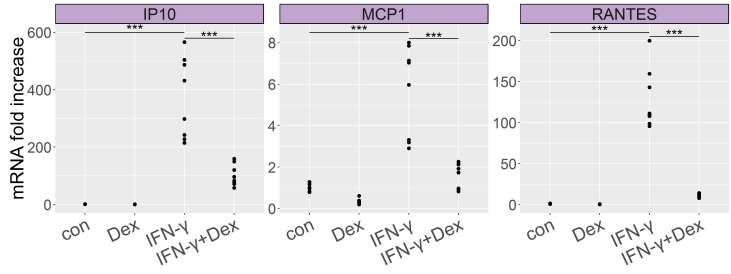
2.3 Results

2.3.1 Lung endothelial cells remain GC sensitive upon stimulation with IFN-γ

IFN-γ plays a crucial role in the induction of chemokines in mouse models of complicated malaria [145, 150]. To evaluate if IFN-γ might cause GC resistance in lung endothelial cells, we stimulated L2 MVECs with IFN-γ in the presence or absence of dexamethasone for 24 hours. IFN-γ induced CCL2 (MCP-1), CXCL10 (IP-10), and CCL5 (RANTES) on both protein and RNA levels (Figure 4). CXCL10 (IP-10) showed the strongest induction. Dexamethasone inhibited IFN-γ-induced chemokine secretion, suppressing CCL5 (RANTES) by 90% and both CXCL10 (IP-10) and CCL2 (MCP-1) approximately by 50% (Figure 4a). Real-time qPCR experiments revealed 90% inhibition of CCL5 (RANTES) and 71% inhibition of both CXCL10 (IP-10) and CCL2 (MCP-1). Moreover, treatment with dexamethasone (with or without IFN-γ) induced MKP-1 (DUSP1), GILZ (Tsc22d3) and FKBP51 (Figure 7), indicating that IFN-γ did not alter GC-mediated transactivation of these genes. These results indicate that IFN-γ-stimulated lung endothelial cells remain GC sensitive.



(a)



(b)

Figure 4: Dexamethasone inhibits pro-inflammatory chemokine secretion in lung endothelium stimulated with IFN-γ. L2 MVECs were treated with vehicle (con) or IFN-γ (20 ng/mL) in the presence or absence of dexamethasone (Dex, 100 nM) for 24 hours. CCL2 (MCP-1), CXCL10 (IP-10) and CCL5 (RANTES) expression levels were analyzed by ELISA (a) and real-time qPCR (b). Statistical significance was evaluated using ANOVA (*** p <0.001). Data show combined results from 3 independent experiments.

2.3.2 GCs differentially affect the transcriptional response to IFN-γ in lung endothelial cells

To further characterize the transcriptional targets indicating GC sensitivity in lung endothelial cells, we analyzed by RNA-seq the transcriptome of L2 MVECs in 4 different conditions: unstimulated control, stimulated with dexamethasone alone, stimulated with IFN-γ alone, stimulated with IFN-γ and dexamethasone. IFN-γ induced expression of various guanylate binding proteins (GBPs), which trigger antimicrobial effector mechanisms via inhibition of

replication of intracellular pathogens (Figure 5a) [155]. Similarly, we found several interferon-induced proteins with tetratricopeptide repeats (IFITs). These proteins inhibit viral replication [156]. IFN-γ also increased expression of H-2 class II histocompatibility genes and adhesion molecules such as VCAM-1 and ICAM-1. Moreover, IFN-γ potently induced transcription of various pro-inflammatory CXC and CCL chemokines including CXCL9 (MIG), CXCL11 (MIG), CXCL10 (IP-10), CCL5 (RANTES) and CCL2 (MCP-1, Figure 5a). These data on chemokine induction further confirm the ELISA and qRT-PCR experiments shown in Figure 4.

Dexamethasone significantly inhibited large clusters of genes induced by IFN-γ, which included several CXC and CCL chemokines (Table 1). In particular, dexamethasone markedly diminished transcription of CCL5 (RANTES, more than 85% reduction), CXCL11 (MIG, more than 80% reduction), CCL4 and CCL3 (more than 75% reduction, Figure 5b). Also, IFN-γ-induced interleukin 1β (IL-1β), IL-27 and IFITs were downregulated by dexamethasone. In contrast, the expression of GBPs, STAT family members and the cytokine IL-15 was resistant to GC-mediated transcriptional inhibition (Table 1).

In addition, we found a large cluster of genes upregulated by GCs. Dexamethasone induced several anti-inflammatory genes in IFN-γ-stimulated endothelial cells including orosomucoid-1 (Orm1, Fold Change 11.3), DEP domain-containing mTOR-interacting protein (DEPTOR, Fold Change 5.3) and GILZ (Tsc22d3, Fold Change 4.6, Figure 5c) – a known GR target gene. These genes were also upregulated upon treatment with dexamethasone alone. Interestingly, another set of genes was repressed by IFN-γ alone, but the expression was restored when dexamethasone was added. These genes include CXCL12, angiopoietin-like protein (Angptl)7, TGF-β2, TLR7 and tumor necrosis factor receptor superfamily member (TNFRSf)21. Altogether, these data indicate that lung endothelial cells are GC sensitive, when stimulated with IFN-γ.

As for the affected pathways, gene ontology (GO) analysis of genes induced by IFN-γ at least 2 fold and inhibited by dexamethasone at least by 50% (Figure 5d) revealed a high prevalence of biological process GO terms related to regulation of immune response and pro-inflammatory signaling pathways such as MAPK (Figure 5e). Several binding partners have been involved in GR-mediated repression of pro-inflammatory genes including AP-1, NF-κB and interferon regulatory factor (IRF)3 [69, 157, 158]. Interestingly, a computational analysis of the 400-bp region upstream of genes induced by IFN-γ at least 2 fold and inhibited by dexamethasone at least by 50% showed a significant enrichment of the binding sites for IRF and NF-κB families but not for GR (Table 2). These results remain in line with previous findings since in LPS-stimulated macrophages, less than 6% of GR binding sites occurred at proximal promoter regions [158]. Also, GR can tether to DNA-bound TFs such as NF-κB without requiring a GRE motif.

2.3.3 *Pb*NK65 extract in combination with IFN-γ induces GC resistance in lung endothelium

Although GC resistance has been observed in our murine model of MA-ARDS [8], the above results indicate that IFN-γ-stimulated lung endothelial cells remain GC sensitive. As sequestering parasites release a variety of products which can further activate endothelial cells [10, 11], we investigated whether addition of parasite extract might alter the GC sensitivity of L2 MVECs. *Pb*NK65 extract in combination with IFN-γ increased the mRNA levels of CCL2 (MCP-1) and CCL5 (RANTES) respectively 2-fold and 3-fold in comparison to IFN-γ alone (Figure 6a). *Pb*NK65 alone increased only CCL2 (MCP-1) protein levels and interestingly, this induction was sensitive to dexamethasone (Figure 6b). Importantly, dexamethasone failed to inhibit mRNA and protein induction of CCL2 (MCP-1), CCL5 (RANTES) and CXCL10 (IP-10), when cells were chal-

Gene symbol	IFN-γ vs con Log2FC	IFN-γ+Dex vs IFN-γ Log2FC
CXCL16	2.4	-1.2
CXCL14	-1.7	0.75
CXCL12	-0.8	1.2
CXCL11	7.5	-2.6
CXCL10	7.4	-1.6
CXCL9	9.7	-1.6
CXCL5	NS	-1.4
CXCL1	NS	-1.2
CCL27a	0.8	NS
CCL22	3.9	-2
CCL20	1.4	-1.5
CCL17	NS	-1
CCL12	3.8	-1.4
CCL11	1.9	NS
CCL9	-1.3	0.9
CCL8	3.3	-0.9
CCL7	1.6	-1.4
CCL6	1.2	NS
CCL5	6	-3.1
CCL4	2.4	-2.2
CCL3	1.1	-2.2
CCL2	2.2	-1.6
IL-34	NS	-1.1
IL-33	-1.5	NS
IL-27	3.4	-1.8
IL-18	0.6	0.6
IL-15	2.4	-0.5
IL-10	1.4	1.4
IL-7	1.2	NS
IL-6	0.8	-1.6
IL-1α	2.5	-1.6
IL-1β	4.2	-2.5
TNF	2.2	-1.6
TNFSF18	1	NS
TNFSF15	2.1	-1.6
TNFSF13b	2.8	NS
TNFSF11	-0.8	NS
TNFSF8	-1.3	NS

Table 1: Differentially expressed chemokines and cytokines in lung endothelial cells stimulated with IFN-γ in the presence or absence of dexamethasone (FC – fold change, NS – non-significant).

Rank	Motif / Name	p-value	q-value	% of target sequences with motif
1	 IRF8(IRF)/BMDM-IRF8-ChIP-Seq(GSE77884)	1e−13	< 1e−4	40.26%
2	 IRF3(IRF)/BMDM-Irf3-ChIP-Seq(GSE67343)	1e−13	< 1e−4	36.36%
3	 IRF1(IRF)/PBMC-IRF1-ChIP-Seq(GSE43036)	1e−11	< 1e−4	25.97%
4	 PU.1:IRF8(ETS:IRF)/pDC-Irf8-ChIP-Seq(GSE66899)	1e−9	< 1e−4	27.27%
5	 ISRE(IRF)/ThioMac-LPS-Expression(GSE23622)	1e−8	< 1e−4	15.58%
6	 PU.1-IRF(ETS:IRF)/Bcell-PU.1-ChIP-Seq(GSE21512)	1e−7	< 1e−4	63.64%
7	 PRDM1(Zf)/Hela-PRDM1-ChIP-Seq(GSE31477)	1e−7	< 1e−4	35.06%
8	 IRF2(IRF)/Erythroblas-IRF2-ChIP-Seq(GSE36985)	1e−4	0.0013	14.29%
9	 NFkB-p65-Rel(RHD)/ThioMac-LPS-Expression(GSE23622)	1e−4	0.0014	10.39%
10	 NFkB-p65(RHD)/GM12787-p65-ChIP-Seq(GSE19485)	1e−3	0.004	28.57%

Table 2: Top 10 enriched transcription factor-binding motifs in -400 to +100 bp in genes induced by IFN-γ at least 2 fold and repressed by dexamethasone at least by 50%.

lenged with the combination of *Pb*NK65 extract and IFN-γ (Figure 6a and 6b). These results indicate that *Pb*NK65 extract impairs GC-mediated transrepression of these inflammatory chemokines observed after stimulation with IFN-γ alone. In contrast, *Pb*NK65 extract failed to affect GC-induced transactivation of MKP-1 (DUSP1), GILZ (Tsc22d3) and FKBP51 (Figure 7).

To evaluate the time course of the development of GC resistance, we stimulated lung endothelial cells with *Pb*NK65 extract and IFN-γ for 6, 24 and 48 hours in the presence or absence of dexamethasone and analyzed CCL2 (MCP-1) and CXCL10 (IP-10) secretion. After 6 hours chemokine levels remained low both in the resistant condition with *Pb*NK65 extract and IFN-γ and in the sensitive one with IFN-γ alone (Figure 8). GC resistance for CCL2 (MCP-1) occurred following 24 hour stimulation with *Pb*NK65 extract and IFN-γ and was still present after 48 hours. *Pb*NK65 extract and IFN-γ induced GC resistance for CXCL10 (IP-10) already after 6 hours. Notwithstanding these gene-specific differences in kinetics, all studied genes underwent GC resistance upon combining IFN-γ with *Pb*NK56 extract. In contrast, upon stimulation with IFN-γ alone endothelial cells remained fully GC sensitive even after 48 hours.

As a control for the *Pb*NK65 extract, we stimulated lung endothelial cells with extract from non-infected RBCs and evaluated GC sensitivity. RBC extract did not induce CCL2 (MCP-1) or CXCL10 (IP-10) in endothelial cells (Figure 9). When combined with IFN-γ, RBC extract failed to enhance secretion of pro-inflammatory chemokines. Furthermore, dexamethasone inhibited CCL2 (MCP-1) and CXCL10 (IP-10) production induced by IFN-γ in the presence of RBC extract, showing that RBC extract is not able to induce GC resistance. These results confirm that parasite components but not RBC components mediate GC resistance.

2.4 Discussion

Changes to the microvasculature are among the hallmarks of malaria pathology. IFN-γ synergizes with lymphotoxin-α and TNF to induce the expression of adhesion molecules (E-selectin and ICAM-1) in brain endothelial cells [28]. In line herewith, our RNA-Seq analysis in lung endothelial cells showed that IFN-γ induces transcription of VCAM-1 and ICAM-1. Dexamethasone significantly inhibited expression of these adhesion molecules. Moreover, dexamethasone induced protective molecules including anti-inflammatory factors and junctional proteins.

Several cytokines have been shown to induce GC resistance in various cell types. In human bronchial epithelial cells, pre-incubation with IL-17A impairs the inhibitory effect of GCs on TNF-induced IL-8 via PI3K activation and subsequent reduction of HDAC2 activity. However, IL-17A lacks effect on GR-mediated transactivation [159]. In contrast, TGF-β impairs GR-mediated induction of anti-inflammatory genes in A549 cells [160]. IL-13 has been shown to decrease GR ligand binding affinity in monocytes [161]. In PBMCs, IL-17 and IL-23 cause GC resistance in PBMCs via induction of GRβ [162]. Also, IL-2 might cause GC resistance in PMBCs [163]. In eosinophils IL-2 and IL-4 as well as TNF and IFN-γ, or IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5 alone diminish GC-induced apoptosis and therefore cause GC resistance. IL-2 and IL-4 impair GR phosphorylation. Impaired GR phosphorylation correlates with increased protein phosphatase 5 (PP5) activity and PP5 silencing restores GC sensitivity [164].

The involvement of IFN-γ in GC resistance has been extensively studied [165, 166]. In the present study, we show that lung endothelial cells remain GC sensitive upon challenge with IFN-γ. However, combined treatment with IFN-γ and *Pb*NK65 extract impairs GC-mediated inhibition of the production of pro-inflammatory cytokines. IFN-γ has been shown to induce GC

resistance in other disease models in combination with bacterial products or cytokines. For example, cooperative signaling between IFN-γ and LPS induces IL-27 in mouse macrophages and inhibits GR nuclear translocation [165]. In airway smooth muscle cells, treatment with IFN-γ and TNF causes GC resistance. Short term stimulation with IFN-γ and TNF impairs GR binding to DNA and GRE-dependent transcription via upregulation of GRβ, whereas long term treatment depletes GRIP-1 from the GR transcriptional regulatory complexes [166, 167, 168]. In contrast, Goleva and colleagues showed that IFN-γ reverses GC resistance induced in T cells by long term treatment with IL-2 and IL-4 [169].

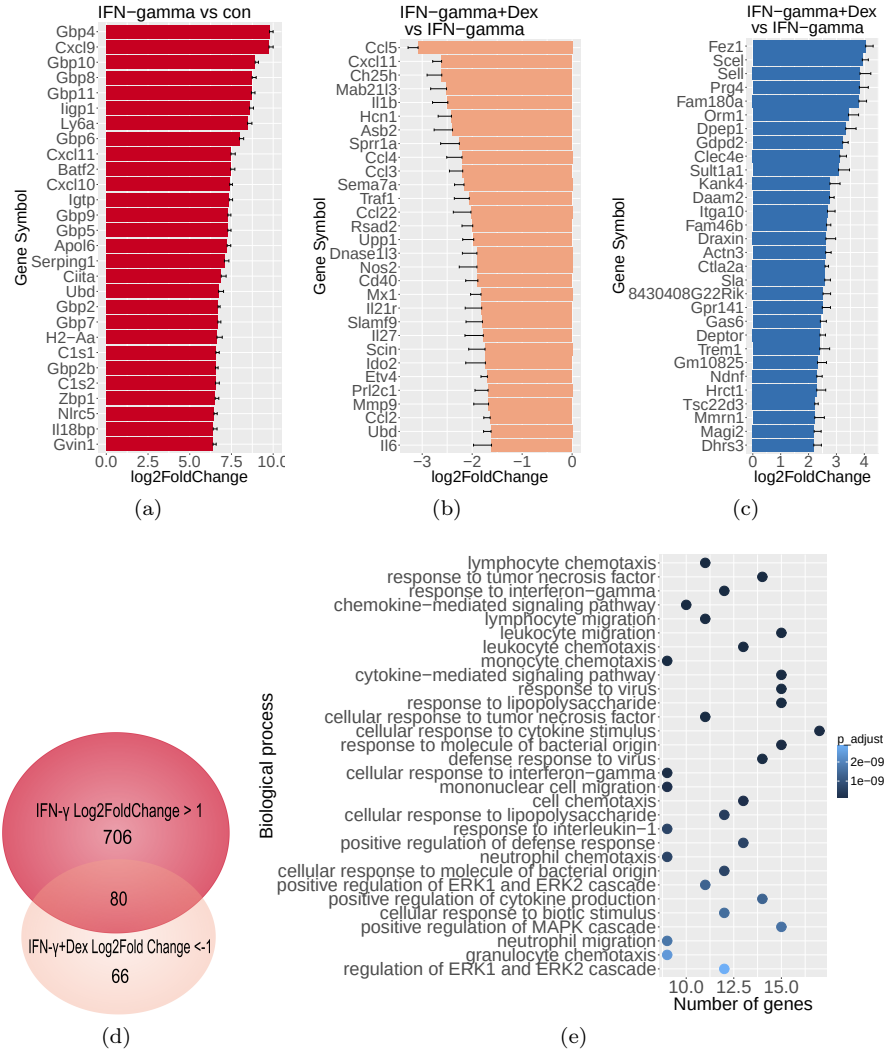
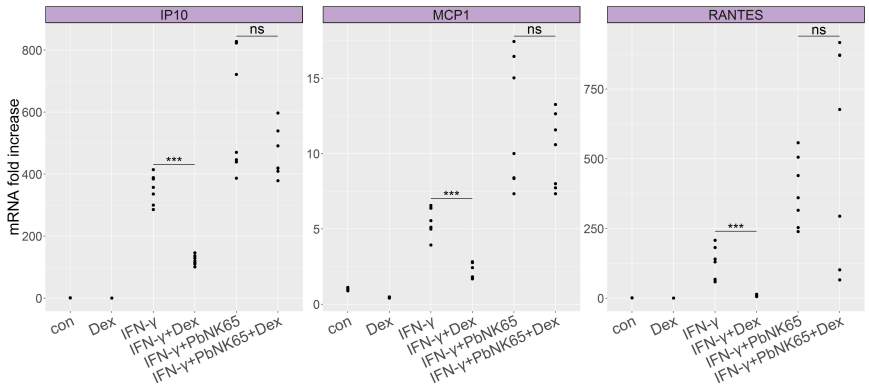
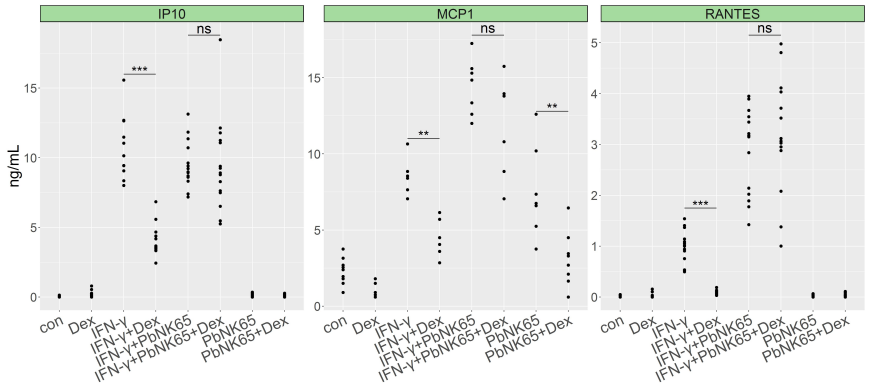


Figure 5: GCs diminish transcriptional activity induced by IFN-γ. Genome wide expression was measured (n=3), log2FoldChange has been depicted including standard error estimate (lfcSE, error bars). (a) Top 30 genes activated by IFN-γ in endothelial cells. (b) Top 30 genes down-regulated by dexamethasone in endothelial cells stimulated with IFN-γ. (c) Top 30 genes induced by dexamethasone in IFN-γ-stimulated endothelial cells. (d) Venn diagram depicts overlap between genes induced by IFN-γ and repressed by dexamethasone (e) Biological process GO analysis (top 30 terms) of genes induced at least two fold by IFN-γ and repressed at least 50% by dexamethasone.



(a)



(b)

Figure 6: *Pb*NK65 extract in combination with IFN-γ induces GC resistance in lung endothelial cells. L2 MVECs were treated with vehicle (con), IFN-γ (20 ng/mL), *Pb*NK65 extract or IFN-γ and *Pb*NK65 extract (PbNK65, 10^7 infected RBCs/mL) in the presence or absence of dexamethasone (Dex, 100 nM) for 24 hours. CCL2 (MCP-1), CXCL10 (IP-10) and CCL5 (RANTES) levels were analyzed by real-time qPCR (a) and ELISA (b). Statistical significance was evaluated using Mann-Whitney test (** p <0.01, *** p <0.001). Data represent combined results from at least 2 independent experiments.

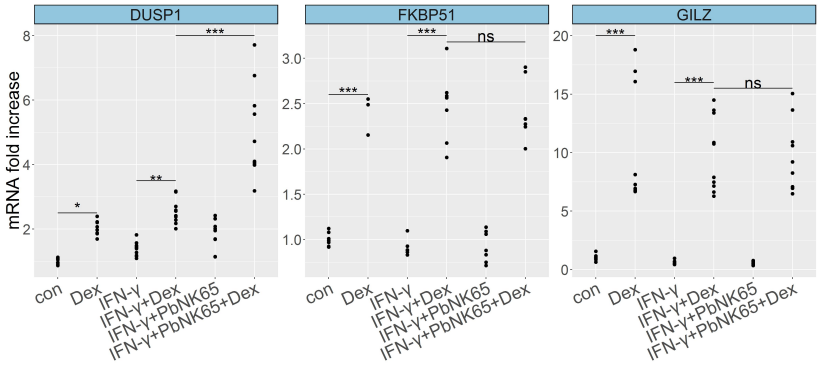


Figure 7: Dexamethasone induces MKP-1 (DUSP1), FKBP51 and GILZ in IFN-γ or IFN-γ and *Pb*NK65-treated lung endothelial cells. L2 MVECs were stimulated with vehicle (con), IFN-γ (20 ng/mL) or IFN-γ and *Pb*NK65 extract (*Pb*NK65, 10^7 infected RBCs/mL) in the presence or absence of dexamethasone (Dex, 100 nM) for 24 hours. MKP-1 (DUSP1), FKBP51 and GILZ expression was analyzed by qRT-PCR. Statistical significance was evaluated using ANOVA (* p <0.05, ** p <0.01, *** p <0.001). Data represent combined results from at least 3 independent experiments.

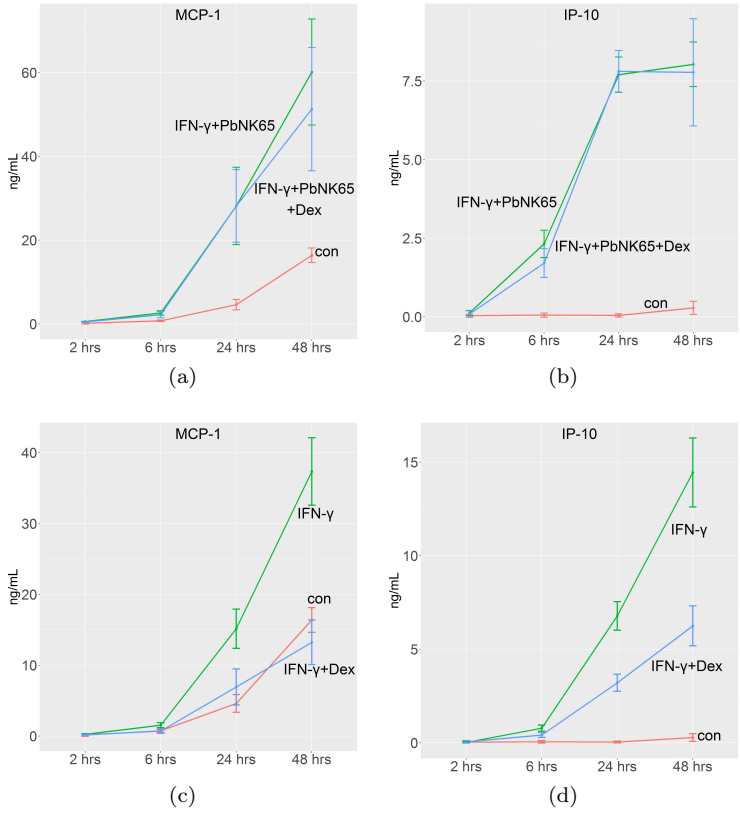


Figure 8: *Pb*NK65 extract in combination with IFN-γ induces GC resistance after 6 or 24 hours. L2 MVECs were stimulated with vehicle (con), IFN-γ (20 ng/mL) and IFN-γ and *Pb*NK65 extract (*Pb*NK65, 10^7 infected RBCs/mL) in the presence or absence of dexamethasone (Dex, 100 nM). CCL2 (MCP-1) and CXCL10 (IP-10) levels in culture supernatant were analyzed by ELISA. Data are presented as mean of 2 independent experiments +/- SD.

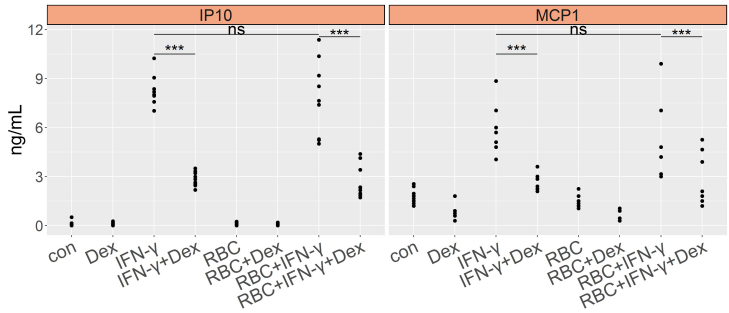


Figure 9: Murine RBC extract exhibits no effect on cytokine induction and GC sensitivity of lung endothelial cells. L2 MVECs were stimulated with vehicle (con), IFN-γ (20 ng/mL), RBC (10^7 RBCs/mL), RBC and IFN-γ in the presence or absence of dexamethasone (Dex, 100 nM) for 24 hours. CCL2 (MCP-1) and CXCL10 (IP-10) production was analyzed by ELISA. Statistical significance was evaluated using ANOVA (*** p <0.001). Data represent combined results from 2 independent experiments.

3 *Pb*NK65 and IFN-γ do not impair GR phosphorylation and translocation in lung endothelial cells

3.1 Introduction

In the previous chapter, I showed that L2 MVECs remain GC sensitive upon stimulation with IFN-γ, but they become GC resistant when IFN-γ is combined with the parasite extract. In this chapter, the mechanism underlying this GC resistance is investigated. The nuclear translocation of GR, which is regulated by phosphorylation, is crucial for GR activity and is often impaired under GC resistant conditions [127].

Ligand binding induces GR phosphorylation (with the exception of serine 150 threonine 159) and therefore GR represents a target for various kinases [170]. For example, cyclin dependent kinase (CDK) complexes and MAPK have been shown to mediate GR phosphorylation. In rats, CDK complexes phosphorylate serine 224 and 232, whereas MAPK modify threonine 171 and serine 246. CDK-mediated phosphorylation is required for full transcriptional activity of GR. In contrast, MAPK-induced phosphorylation of threonine 171 and serine 246 reduces GR transcriptional activity of GR [171]. Interestingly, p38 MAPK has also been shown to induce serine 211 (S211) phosphorylation of GR and GC-mediated apoptosis in lymphoid cells [172]. S211 phosphorylation correlates with transcriptional activity of GR. Phosphorylated S211 has also been used as a biomarker for activated GR since phosphorylated S211 GR mainly localizes in the nucleus [173]

JNK, another member of MAPK family, has been shown to phosphorylate GR at S246. Activation of JNK attenuates GR transcriptional activity, providing a means to terminate GR-mediated transcription when it interferes with

pro-inflammatory signals [174]. Moreover, activated JNK also phosphorylates GR on Serine 226 and enhances GR nuclear export leading to termination of GR transcriptional activity [175]. In this chapter, we investigated the S211 and S226 GR phosphorylation and GR nuclear translocation in lung endothelial cells stimulated with IFN-γ, IFN-γ and *Pb*NK65 in the presence or absence of dexamethasone.

3.2 Materials and methods

3.2.1 Western blot

For Western blot analysis protein extracts from L2 MVECs stimulated with IFN-γ, *Pb*NK65 extract, IFN-γ and *Pb*NK65 extract in the presence or absence of dexamethasone were separated on SDS PAGE gels and blotted onto a PVDF membrane. Blocking was performed with BSA (Carl Roth Gmbh, Belgium) or non-fat dry milk (Bio Rad, USA). The following primary Ab were used p-GR S211 (1:1000, Cell Signaling Technology), p-GR S226 (1:1000, Abcam), GR H300 (1:1000, Santa Cruz, Germany). Fusion solo S system (Vilber, France) was used to produce chemiluminescence Western blot images. Quantification of Western blot images was performed by densitometry (ImageJ software was used).

3.2.2 GR nuclear translocation

Cells were seeded on coverslips and incubated in phenol-red-free and serum-free medium for 4h. Cell fixation, methanol permeabilization and staining were performed according to Cell Signaling guidelines. GR was visualized with the GR polyclonal (H300) antibody (Santa Cruz, Germany), used at 1:200, followed by probing with Alexa Fluor 488 (Invitrogen, Belgium). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) staining. A motorized inverted IX81 FluoView FV1000 laser scanning confocal microscope (Olympus) was used

to record high-resolution images. Assessment of intracellular localization of protein signal was done double-blind.

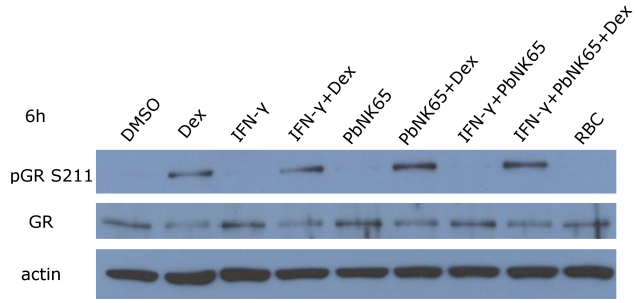
3.3 Results

3.3.1 *Pb*NK65/IFN-γ cotreatment preserves homologous GR downregulation and GR nuclear translocation

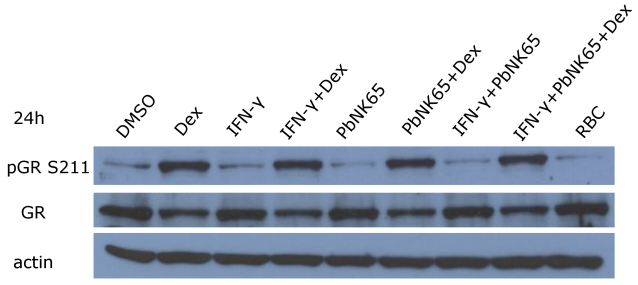
GR undergoes homologous downregulation when incubated with its ligand for longer periods of time. Perturbations of this process may lead to GC resistance [176, 177]. To evaluate the levels of GR, we stimulated L2 MVECs for 6 or 24 hours with vehicle (DMSO), RBC extract, IFN-γ, *Pb*NK65 extract, IFN-γ and *Pb*NK65 extract in the presence or absence of dexamethasone. The levels of GR remained unchanged in the resistant condition with IFN-γ and *Pb*NK65 extract when compared to the sensitive condition with IFN-γ alone. Additionally, the capacity and extent of GC-induced GR homologous downregulation remained unaffected in either the sensitive or resistant condition. We also investigated the S211 phosphorylation of GR, since this modification is associated with transcriptionally active GR and provides a means for cross-talk with other signaling pathways [178]. As expected, dexamethasone alone induced S211 phosphorylation. Moreover, phosphorylation of GR at S211 remained present when cells were challenged with IFN-γ or IFN-γ with *Pb*NK65 extract in the presence of dexamethasone (Figure 10a and 10b). We also evaluated the GR S226 phosphorylation, which inhibits GR function [174, 175]. Dexamethasone inhibited GR S226 phosphorylation upon stimulation with IFN-γ or IFN-γ with *Pb*NK65 extract (Figure 10c). These data indicate that GC resistance following IFN-γ/*Pb*NK65 cotreatment is most likely not caused by defective GR phosphorylation or GR homologous downregulation mechanisms.

Since impaired GR nuclear translocation leads to GC resistance [169, 127], we assessed the ability of dexamethasone to induce GR translocation in lung

endothelial cells. L2 MVECs were challenged for 24 hours with *Pb*NK65 extract and IFN-γ and dexamethasone was added during the last hour of stimulation (Figure 11). Immunofluorescence microscopy revealed that in unstimulated cells GR localized mainly in the cytoplasm and translocated to the nucleus upon dexamethasone exposure. GR also translocated to the nucleus upon treatment with IFN-γ and dexamethasone. Furthermore, the translocation occurred in the GC resistant condition with *Pb*NK65 extract and IFN-γ (Figure 11). We also obtained similar data for a shorter time point of 2 hours or with 24 hour co-treatment with IFN-γ, *Pb*NK65 extract and dexamethasone (Figure 12). These data indicate that GC resistance is not caused by any defect in GR translocation.



(a)



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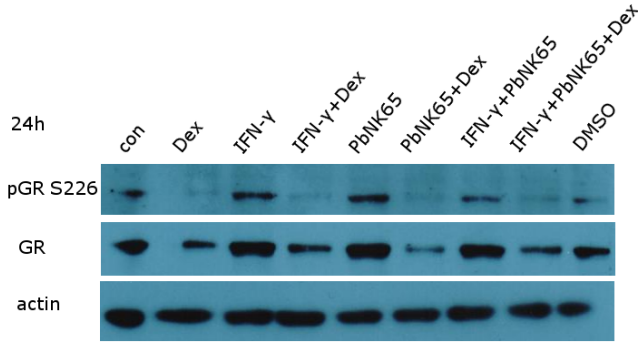


(c)

Figure 10: IFN-γ and *Pb*NK65 extract do not interfere with either GR expression or GR phosphorylation. (a, b, c) Western blot analysis of lysates of L2 MVECs stimulated for 6 or 24 hours with solvent (DMSO), red blood cells extract (RBC, 10^7 RBCs/mL), IFN-γ (20 ng/mL), *Pb*NK65 extract (10^7 infected RBCs/mL), IFN-γ and *Pb*NK65 extract in the presence or absence of dexamethasone (Dex, 100 nM).

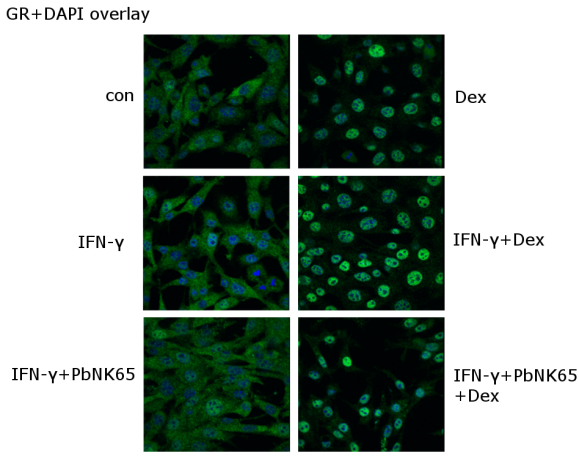


Figure 11: IFN-γ and *Pb*NK65 extract preserve GR nuclear translocation in lung endothelial cells. L2 MVECs were stimulated with solvent (con), IFN-γ (20 ng/mL) or IFN-γ and *Pb*NK65 extract (10^7 infected RBCs/mL) for 24 hours and treated for 1 hour with dexamethasone (100 nM). Endogenous GR was visualized (green) through indirect immunofluorescence using anti-GR Ab. DAPI staining (blue) indicates the nuclei of the cells.

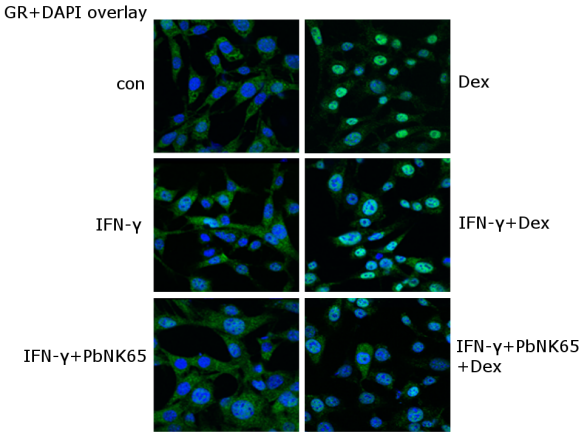


Figure 12: IFN-γ and *Pb*NK65 do not interfere with GR expression after 2 hours. L2 MVEC cells were stimulated with solvent (con), IFN-γ (20ng/mL) or IFN-γ and *Pb*NK65 extract (10^7 infected RBCs/mL) for 2 hours and treated for 1 hour with dexamethasone (Dex, 100nM). Endogenous GR was visualized (green) through indirect immunofluorescence using anti-GR Ab. DAPI staining (blue) indicates the nuclei of the cells.

3.4 Discussion

Various mechanisms leading to GC resistance have been proposed. We show here that treatment with IFN-γ and *Pb*NK65 extract, in the presence or absence of GCs, affects neither GR levels nor GR S211 or S226 phosphorylation in lung endothelial cells. GR S211 phosphorylation is associated with enhanced GR activity whereas GR S226 phosphorylation exerts inhibitory effects on GR [170, 174, 175]. In contrast to our findings, several pro-inflammatory cytokines have been shown to reduce GR levels or GR S211 phosphorylation. For example, IL-2 and IL-4 impair GR S211 phosphorylation in T cells [169]. TNF downregulates the levels of hepatic GR *in vivo* and TGF-β exposure (before challenge with IL-1α) reduces GR levels in A549 cells [179, 160]. However, TGF-β induced by respiratory syncytial virus fails to downregulate GR levels in human airway epithelial cells [180]. Proteasomal degradation of GR reduces its levels and was proposed to cause GC resistance in endothelial cells [135, 136].

Impaired GR nuclear translocation represents another way to mediate GC resistance in various models. In B cells, treatment with IL-4 and IL-15 inhibits GR translocation [127]. Similarly, IL-4 and IL-2 impair GR translocation in T cells [169]. Superantigens block GR translocation in PBMCs [181]. In contrast, GR translocation remains functional in GC resistant HUVECs and lack of response to GCs is associated with defects downstream of GR translocation [139]. This is in line with our data, since GR still translocated in the resistant condition with IFN-γ and *Pb*NK65 extract. Moreover, IFN-γ and *Pb*NK65 extract did not affect mRNA levels of FKBP51 (Figure 7), which sequesters GR in the cytoplasm and has been implicated in GC resistance [182, 183].

4 Differential effects of GCs on STAT1 and MAPK signaling in lung endothelial cells challenged with IFN-γ or *Pb*NK65 and IFN-γ

4.1 Introduction

In this chapter, I investigated the effects of GCs on IFN-γ-induced signaling. IFN-γ is a widely expressed cytokine that signals through the IFN-γ receptor (IFNGR), which is composed of two subunits: IFNGR1 and IFNGR2 [184]. Each of the subunits associates with a specific member of the Janus activated kinase (JAK) family. IFNGR1 interacts with JAK1 while IFNGR2 associates with JAK2 [185]. The JAK-STAT signaling pathway was discovered as the first pathway activated by IFNs. This pathway involves nuclear translocation of STATs and induction of gene transcription. Upon IFN-γ binding to its receptor, autophosphorylation of JAK1 and JAK2 occurs. Subsequently, JAK1 and JAK2 phosphorylate STAT1, which form homodimers and translocate to the nucleus to activate gene transcription (Figure 13) [186].

IFN-γ has also been shown to activate the MAPK pathway (Figure 13) [187]. The mammalian family of MAPKs consists of extracellular signal-regulated kinase (ERK), p38, and JNK. Each member of the MAPK family has several isoforms: ERK1 to ERK8; p38-α,-β,-γ, and -δ; and JNK1 to JNK3 [188]. The MAPK pathway is activated by sequential phosphorylation starting with phosphorylation of MAPK kinases (MAPKKs) by MAPK kinase kinases (MAPKKKs) [189]. MAPKs control various processes such as proliferation, differentiation and survival and diverse stimuli can activate this pathway including cytokines, hormones, growth factors and cellular stressors. Activated MAPKs phosphorylate diverse target proteins including kinases, phosphatases, transcription factors and cytoskeletal proteins [188]. Due to the role of MAPK in

the regulation of essential cellular processes, this pathway has been implicated in the development of various diseases such as cancer and neurodegenerative diseases [190].

The ERK pathway is the best studied MAPK pathway and its activation by growth factors and mitogens is relevant in cancer. Constitutive activation of this pathway in cancer usually occurs at the early steps of the cascade. Moreover, the activity of ERK target transcription factors such as myc and AP-1 becomes amplified [188]. Both JNK and p38 are activated by cytokines (for example TNF and IL-1β) and environmental stresses [190, 188, 191]. JNK1 and 2 are ubiquitously expressed whereas JNK3 is expressed only in heart, brain and testis. JNK activation requires dual phosphorylation on tyrosine and threonine residues. Upon activation, JNK and p38 translocate to the nucleus and interact with their target transcription factors [188].

Since the MAPK pathway is activated by phosphorylation, dephosphorylation seems the most efficient way to switch off MAPK signaling. Several phosphatases have been shown to inactivate MAPK pathway including tyrosine, serine/threonine and dual-specificity MAPK phosphatases (MKPs or DUSPs). In mammals, at least ten MKPs have been described with MKP-1 (DUSP1) being the best studied member of MKP family. MKPs affect all aspects of immune response dependent on MAPK pathway, for example induction of inflammatory mediators such as TNF, IL-1β, IL-6 and prostaglandin [192]. MKP-1 (DUSP1) plays a crucial role in the inhibition of JNK and p38 signaling and attenuates inflammatory cytokine production in macrophages challenged with LPS [193]. Similarly, MKP-1 (DUSP1) KO mice exhibit increased mortality and cytokine production upon challenge with LPS. MKP-1 (DUSP1) KO macrophages showed prolonged p38 activation and enhanced cytokine production upon challenge with LPS [194].

IFN-γ has been shown to activate all members of MAPK pathway. In

macrophages, IFN-γ stimulation results in strong activation of p38 at early time points and weak activation of ERK and JNK at later time points. p38 is required for CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and TNF induction [187]. Another study showed that IFN-γ activates the ERK pathway and induces gene transcription dependent on C/EBP-B. Interestingly, this transcription requires STAT1 but is JAK1 independent [195]. Matsuzawa and colleagues identified a link between p38 signaling and autophagy induction by IFN-γ in macrophages. In contrast, this induction is STAT1 independent [196].

To elucidate the mechanisms of the inhibitory actions of GCs on the IFN-γ stimulated endothelial cells and to clarify the *Pb*NK65-induced GC resistance, the effects of GCs on the STAT1 and MAPK pathways were investigated.

4.2 Materials and methods

4.2.1 ELISA

Supernatants from L2 MVECs stimulated for 24 hours with the combinations of murine IFN-γ (20 ng/mL, PeproTech, USA), parasite extract (10^7 infected RBCs/mL) and JNK, p38 and ERK inhibitors (SP600125, SB203580 and FR180204; R&D, UK, used at 20 μM, 5 μM and 10 μM, respectively) were stored at -20°C. Cytokines were analyzed by ELISA (R&D) according to the manufacturer's protocol.

4.2.2 Western blot

For Western blot analysis protein extracts from L2 MVECs stimulated with IFN-γ, *Pb*NK65 extract, IFN-γ and *Pb*NK65 extract in the presence or absence of dexamethasone were separated on SDS PAGE gels and blotted onto a PVDF membrane. Blocking was performed with BSA (Carl Roth Gmbh, Belgium) or non-fat dry milk (Bio Rad, USA). The following primary Ab were used for staining of JNK, pJNK, p38, p-p38 (1:2000, Cell Signaling Technology, The

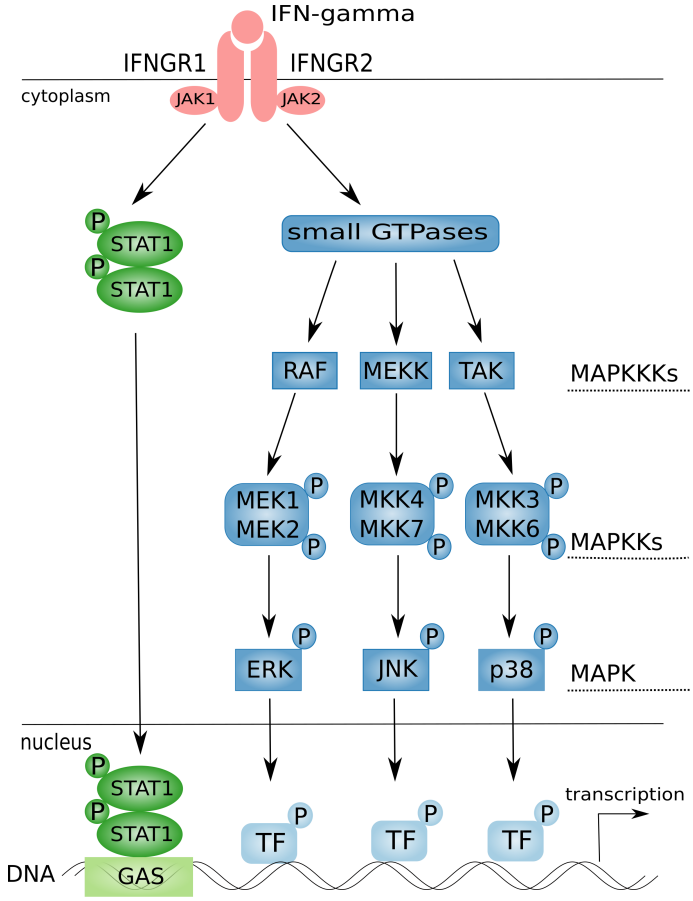


Figure 13: IFN-γ signals through STAT1 and MAPK pathways. IFN-γ binds a cell-surface receptor called type II IFN receptor. This receptor consists of two subunits, IFNGR1 and IFNGR2. Upon phosphorylation, STAT1 homodimer translocates to the nucleus, binds to IFN-γ-activated site (GAS) and activates gene transcription. Binding of IFN-γ to its receptor also induces small GTPases to swap their GDP for a GTP. This results in MAPKKKs activation, which activates MAPKKs. The last step of this cascade is MAPK activation. Activated MAPKs translocate to the nucleus and phosphorylate target transcription factors (TF) to induce gene expression.

Netherlands), p-STAT1 Tyr701 (1:1500, Cell Signaling Technology) and STAT1 (1:5000, Cell Signaling Technology). Fusion solo S system (Vilber, France)

was used to take chemiluminescence Western blot images. Quantification of Western blot images was performed by densitometry (ImageJ software was used).

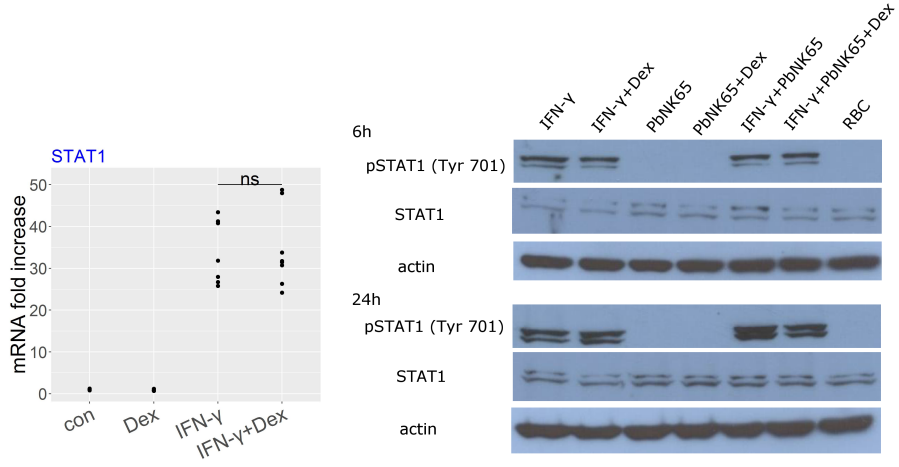
4.2.3 Quantitative RT-PCR

RNA was extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were evaluated with Nanodrop 1000 (Thermo Scientific, Belgium). RNA (0.25μg) was converted to cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR was performed with 6.25 or 0.125 ng cDNA using predesigned primers (IDT) and TaqMan Universal PCR Master Mix (Applied Biosystems), respectively.

4.3 Results

4.3.1 Dexamethasone fails to inhibit STAT1 activation upon challenge with IFN-γ or *Pb*NK65 extract and IFN-γ

Since STAT1 is involved in gene induction by IFN-γ, we evaluated the effect of dexamethasone on STAT1 activation in the GC sensitive condition with IFN-γ and in the resistant one with IFN-γ and *Pb*NK65 extract. RNA-Seq data showed that dexamethasone failed to affect STAT1 expression induced by IFN-γ and this result was validated by qRT-PCR (Figure 14a). Since STAT1 phosphorylation at Tyr 701 controls STAT1 signaling, we subsequently addressed the impact of dexamethasone on phosphorylated STAT1. We showed that STAT1 phosphorylation induced by IFN-γ remained unaffected by dexamethasone both in the GC sensitive (IFN-γ) and the GC resistant condition (IFN-γ and *Pb*NK65 extract, Figure 14b).



(a) (b)

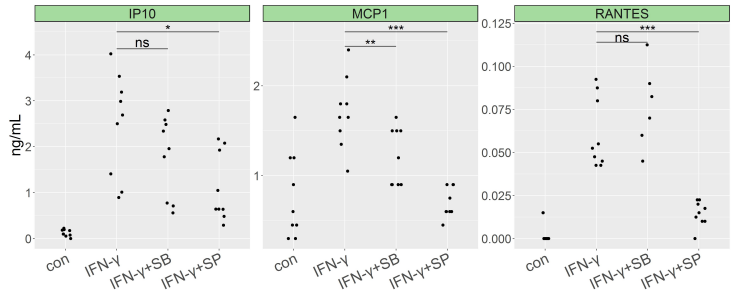
Figure 14: Dexamethasone failed to inhibit STAT1 expression or STAT1 phosphorylation induced by IFN-γ or IFN-γ and *Pb*NK65 extract. (a) L2 MVECs were stimulated with solvent (con) and IFN-γ (20 ng/ mL) in the presence or absence of dexamethasone (Dex, 100 nM) for 24h. STAT1 expression was analyzed by qRT-PCR. Statistical significance was evaluated using Mann-Whitney test. Data show combined results from 3 independent experiments. (b) Western blot analysis of lysates of L2 MVECs stimulated for 6 or 24 hours with IFN-γ (20 ng/mL), *Pb*NK65 extract (10^7 infected RBCs/mL), IFN-γ and *Pb*NK65 extract in the presence or absence of dexamethasone (Dex, 100nM) and red blood cells extract (RBC, 10^7 RBCs/mL) was performed using anti-STAT1 and anti-pSTAT1 Ab.

4.3.2 IFN-γ and *Pb*NK65 extract impair GC-mediated inhibition of MAPK signaling

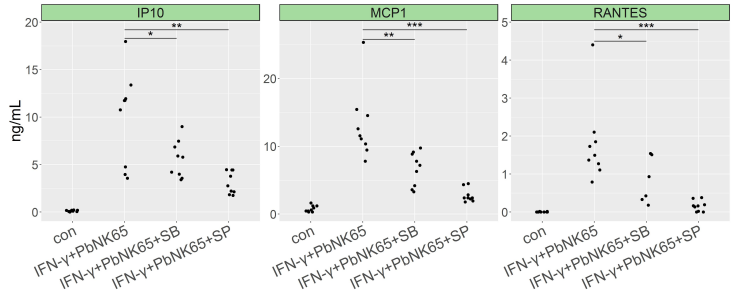
MAPK family members play an important role in the generation and fine-tuning of inflammatory responses and are known to be activated by IFN-γ receptor signaling [187, 196]. To address the role of specific members of the MAPK family in the induction of pro-inflammatory chemokines in our model, we used the JNK, p38 and ERK specific inhibitors: SP600125, SB203580 and FR180204, respectively. Treatment with JNK and p38 inhibitors significantly

reduced pro-inflammatory response induced by IFN-γ in lung endothelial cells. Inhibition of p38 reduced CCL2 (MCP-1) levels by 28% whereas inhibition of JNK blocked CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) by 55%, 59% and 83% respectively (Figure 15a). Accordingly, JNK and p38 inhibition blocked chemokine induction in the GC resistant condition upon combining IFN-γ with *Pb*NK65 extract. SB203580 inhibited CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) levels by 44%, 49% and 54%, whereas SP600125 reduced CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) by 69%, 79% and 94%, respectively (Figure 15b). Inhibition of ERK failed to reduce CXCL10 (IP-10) CCL2 (MCP-1) or CCL5 (RANTES) levels upon challenge with IFN-γ and *Pb*NK65 extract (Figure 16).

Since previous studies indicated that GCs inhibit MAPK family members phosphorylation [94, 197], we investigated the effects of dexamethasone on p38 and JNK phosphorylation in endothelial cells stimulated with IFN-γ with and without *Pb*NK65 extract in the presence or absence of dexamethasone. We found that dexamethasone blocked p38 and JNK phosphorylation upon challenge with IFN-γ (Figure 17a and 17b). Quantification of the Western blot data revealed that, upon challenge with IFN-γ, dexamethasone reduced p38 and JNK phosphorylation on average by 69% and 61%. In unstimulated cells, dexamethasone also blocked p38 and JNK phosphorylation by 58% and 63%, respectively (Figure 17c and 17d). In contrast, when IFN-γ was combined with *Pb*NK65 extract dexamethasone failed to inhibit p38 and JNK phosphorylation by more than 14% and 4% (Figure 17a–17d). These data suggest that dexamethasone inhibits IFN-γ-mediated induction of CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) at least in part by blocking the activation of p38 and JNK. Furthermore, this inhibitory mechanism is impaired in the GC resistant condition.



(a)



(b)

Figure 15: p38 (SB) and JNK (SP) inhibitors impair pro-inflammatory cytokine expression in lung endothelial cells. L2 MVECs were stimulated with IFN-γ (20 ng/mL) (a) or IFN-γ (20 ng/mL) and *Pb*NK65 extract (*Pb*NK65, 10^7 infected RBCs/mL) (b) in the presence of JNK inhibitor SP600125 (SP, 20 μM) or p38 inhibitor SB203580 (SB, 5 μM) for 24 hours. Protein levels of CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) were determined by ELISA. Statistical significance was evaluated using ANOVA (* p <0.05, ** p <0.01, *** p <0.001). Data represent combined results from 3 independent experiments.

4.4 Discussion

Since STAT1 signaling mediates transcription of a significant subset of IFN-γ-induced genes, we investigated the effects of GCs on STAT1 activation. In our model, GCs failed to affect STAT1 expression and phosphorylation. Conversely, GCs suppress STAT1 phosphorylation via SOCS1 induction in macrophages activated with TLR-ligands [198]. Another study showed that GCs inhibit STAT1 expression after long-term incubation in PBMCs stimulated with IFN-γ, but

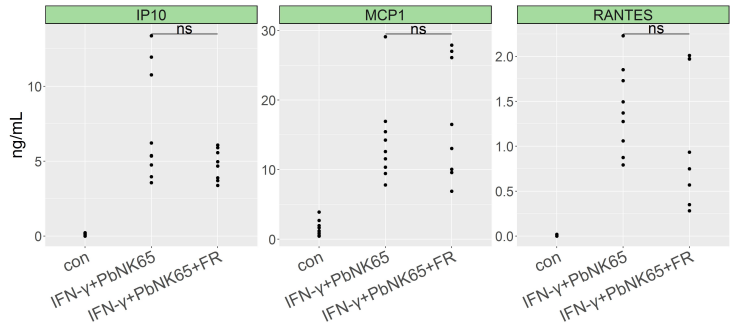


Figure 16: ERK inhibitor (FR) fails to impair pro-inflammatory cytokines expression in lung endothelial cells stimulated with IFN-γ and *Pb*NK65 extract. L2 MVECs were stimulated with IFN-γ (20 ng/mL) and *Pb*NK65 extract (*Pb*NK65, 10^7 infected RBCs/mL) in the presence of ERK inhibitor (FR180204, 10 μM) for 24 hours. Protein levels of CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES) were determined. Statistical significance was evaluated using Mann-Whitney test. Data show combined results from 3 independent experiments.

fail to affect its protein stability [199]. In macrophages challenged with type I IFN, GCs antagonize transcriptional complex ISGF3, which is composed of STAT1, STAT2 and IRF9, via depletion of GR-interacting protein 1 (GRIP1), also known as transcriptional mediators/intermediary factor 2 (TIF2), used by ISGF3 as co-activator [200]. GRIP1/TIF2 mediates anti-inflammatory actions of GCs in macrophages via inhibition of cytokine gene transcription [201]. Furthermore, conditional deletion of GRIP1 in obese mice results in macrophage infiltration and inflammation in the liver [202].

Here, we also studied the effect of dexamethasone on the MAPK signaling pathway – a known target of the anti-inflammatory action of GCs. We found that JNK and p38 play an important role in the induction of pro-inflammatory cytokines in lung endothelial cells upon stimulation with IFN-γ. Dexamethasone inhibited JNK and p38 phosphorylation upon challenge with IFN-γ. Together, these data suggest that inhibition of MAPK signaling is a major mechanism of GC action in these cells. GCs have been shown to block

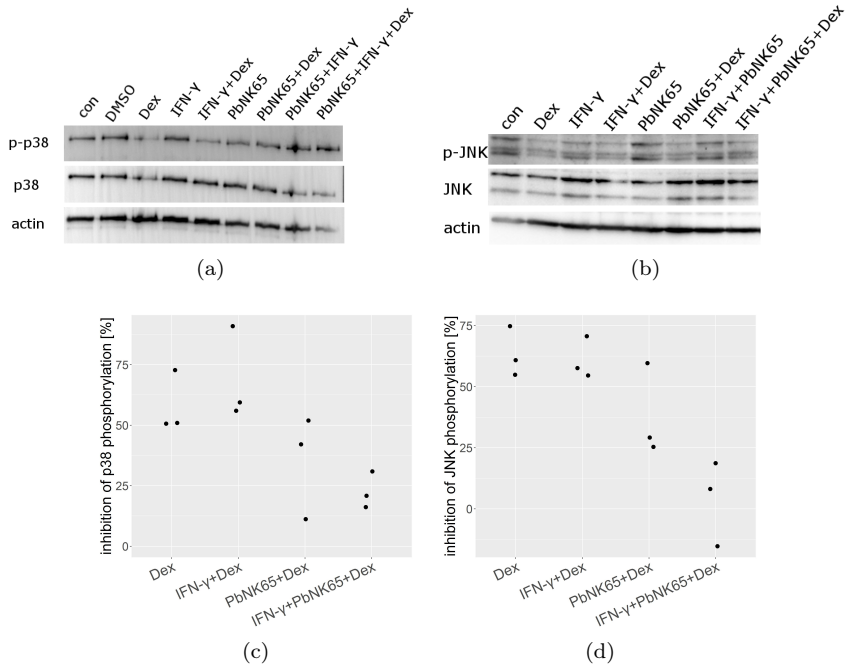


Figure 17: Dexamethasone fails to suppress JNK and p38 activation after challenge with IFN-γ and *Pb*NK65 extract. Western blot analysis of L2 MVEC lysates stimulated for 24 hours with IFN-γ (20 ng/mL), IFN-γ and *Pb*NK65 (10^7 infected RBCs/mL) extract in the presence or absence of dexamethasone (Dex, 100 nM) was performed using anti-p-p38, anti-p38 (a), anti-pJNK and anti-JNK (b) Ab. Data are representative of 3 independent experiments. (c, d) Data from 3 independent experiments were normalized against actin and percentage inhibition by dexamethasone was calculated based on the following comparisons: Dex vs DMSO, IFN-γ+Dex vs IFN-γ, *Pb*NK65+Dex vs *Pb*NK65 and *Pb*NK65+IFN-γ+Dex vs *Pb*NK65+IFN-γ.

MAPK phosphorylation in various experimental models. For example, GCs inhibit JNK via protein-protein interaction in HeLa cells. GR interacts with JNK via a hormone-regulated JNK docking site in the GR ligand-binding domain and induces disassembly of JNK from mitogen-activated protein kinase kinase 7 (MKK7) [197]. In macrophages challenged with LPS, dexamethasone inhibits p38 but neither ERK nor JNK [94]. However, dexamethasone blocks

JNK phosphorylation in HUVECs stimulated with TNF [203]. GCs also inhibit JNK, p38 and ERK phosphorylation in human lung endothelial cells challenged with TNF, IL-1β and H_2O_2 [204].

In this thesis, we show that the inhibitory actions of GCs on MAPK signaling become impaired in lung endothelial cells upon challenge with *Pb*NK65 extract and IFN-γ. Western blot analysis revealed that dexamethasone fails to inhibit p38 and JNK phosphorylation, resulting in sustained inflammatory chemokine expression and GC resistance.

Numerous studies confirm a highly complex interaction between GR and MAPK signaling. In chapter 3, I showed that p-38 MAPK-mediated GR S211 phoshorylation remains unhampered upon challenge with IFN-γ and *Pb*NK65. GR phosphorylation by p38 mediates beneficial effects of GCs. For example, GC-induced apoptosis in lymphoid cells requires S211 phosphorylation of GR by p38 [172]. Similarly, p38 activation by LPS in a model of acute lung injury enhances anti-inflammatory actions of GR [67]. In contrast, JNK negatively regulates the activities of GR by S226 phosphorylation, resulting in enhanced nuclear export and termination of GR signaling [175]. In chapter 3, I found that stimulation with IFN-γ and *Pb*NK65 fails to enhance GR S226 phosphorylation. MAPK and GR interaction might also result in GC resistance. In airway smooth muscle cells, p38 phosphorylates GR at S203 acting as a negative regulator of GR transcriptional activity [205]. Inhibition of JNK enhances GR binding to GREs in mouse hippocampal cells [206]. Moreover, JNK activation by cholesterol impairs GR-mediated transactivation [207].

MKP-1 (DUSP1) is an important mediator of GC inhibitory actions on MAPK signaling [74, 208]. MKP-1 (DUSP1) inhibits p38 in macrophages stimulated with TNF and also blocks JNK and p38 in macrophages challenged with LPS [94, 209]. Furthermore, in macrophages from patients with severe asthma, the activity of p38 MAPK was increased while expression of MKP-1 (DUSP1)

was reduced [126]. MKP-1 (DUSP1) also blocks p38 in endothelial cells [203]. We observed increased mRNA levels of MKP-1 (DUSP1) after stimulation with IFN-γ and *Pb*NK65 compared to IFN-γ alone in the presence of dexamethasone. Moreover, we evaluated the expression of GILZ upon challenge with IFN-γ and *Pb*NK65 and dexamethasone and we found no difference when compared with IFN-γ alone. This indicates that GC-mediated transactivation is not affected by the GC resistance induced by *Pb*NK65. GILZ has been suggested to induce MKP-1 (DUSP1) expression. However, silencing of GILZ (Tsc22d3) in HUVECs failed to alter the pro-inflammatory response to TNF [210, 211].

In conclusion, we here show that *Pb*NK65 extract in combination with IFN-γ impairs GC-mediated transcriptional inhibition of inflammatory chemokines in murine lung endothelial cells. In contrast, lung endothelial cells remain GC sensitive when challenged with IFN-γ alone. GCs block activation of JNK and p38 upon challenge with IFN-γ. However, *Pb*NK65 extract interferes with the inhibitory actions of GCs on p38 and JNK.

5 Conclusion

MA-ARDS remains an often lethal and poorly understood complication of malaria infections [2, 7]. Currently efficient treatment for this syndrome is not available. In an *in vivo* model of MA-ARDS, extremely high doses of GCs were needed to block MA-ARDS. Furthermore, GCs failed to inhibit the expression of pro-inflammatory cytokines and chemokines in the lungs of infected mice, suggesting that malaria can decrease GC sensitivity [8]. In this thesis, we investigated the molecular mechanisms of malaria-induced GC resistance. Since endothelial cells play an important role in malaria, we studied GC sensitivity of lung endothelial cells challenged with *Pb*NK65 and IFN-γ. IFN-γ expression is increased in the lungs of *Pb*NK65-infected mice [8]. NK cells are the earliest source of IFN-γ during both blood and liver stage of malaria. Upon initiation of adaptive immune response T cells become the major producers of IFN-γ [145]. We found that lung endothelial cells remain GC sensitive upon challenge with IFN-γ. In contrast, upon stimulation with *Pb*NK65 and IFN-γ the cells become GC resistant. We found that GCs block p38 and JNK signaling upon challenge with IFN-γ alone. However, when *Pb*NK65 is combined with IFN-γ, GCs fail to block MAPK signaling and GC resistance occurs (Figure 18). Interestingly, GC-mediated transactivation remains unaffected upon challenge with *Pb*NK65 and IFN-γ.

Only a limited number of studies addressed GCs as a potential therapy against complications of malaria. During cerebral malaria, infected RBCs adhere to the endothelium in the brain leading to enhanced expression of adhesion molecules and blood brain barrier damage [6]. GCs exert diverse actions on endothelial cells that could be particularly beneficial in the treatment of cerebral malaria. For example, GCs preserve endothelial barrier integrity via upregulation of junctional proteins such as claudin-5 and VE-cadherin [113, 114, 115]. Moreover, GCs downregulate the expression of MMP-9, an enzyme involved in

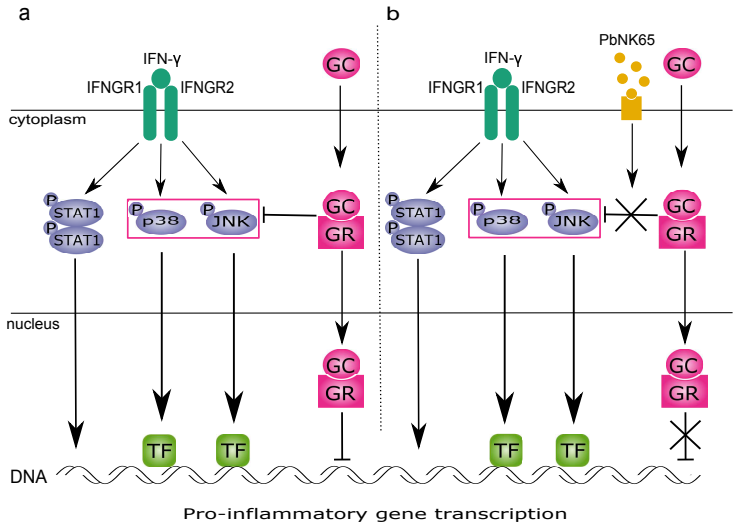


Figure 18: *Pb*NK65 extract in combination with IFN-γ impairs inhibitory effects of GCs on MAPK in lung endothelium. (a) IFN-γ signals through IFNGR and activates STAT1 and MAPK signaling. STAT1 homodimer translocates to the nucleus and activates gene transcription. MAPKs induce gene expression via specific transcription factors (TFs). Upon entering the cell, GC bind to GR and inhibit activation of JNK and p38 induced by IFN-γ. This reduces pro-inflammatory gene expression, including CXCL10 (IP-10), CCL2 (MCP-1) and CCL5 (RANTES). (b) When cells are stimulated with IFN-γ and *Pb*NK65 extract, GCs fail to block p38 and JNK activation. Therefore, transcription of pro-inflammatory genes remains unaffected.

the cleavage of junctional proteins [116, 117, 118]. GC resistance may explain the failure of two clinical trials performed in patients with cerebral malaria [212, 213]. Therefore, it is important to investigate GC resistance in malaria in more detail.

GCs could also be considered as a potential therapy for MA-ARDS. This syndrome is characterized by severe inflammation and massive leukocyte infiltration due to endothelial barrier damage in the lungs [2]. Besides preserving endothelial barrier integrity, several other actions of GCs on endothelium would be beneficial in MA-ARDS. For example, GCs have been shown to downregulate

cytokine and chemokine expression in endothelial cells [98, 97, 103]. This could reduce leukocyte infiltration. Moreover, GCs also inhibit pro-inflammatory transcription factors such NF-κB and AP-1 [96]. The flipside of the coin is the fact that the use of GCs in patients with ARDS remains controversial. Clinical trials provide conflicting data on the timing and dosage of treatment. Short term treatment with high doses of GCs fails to improve the outcome of patients with ARDS. Prolonged treatment with low doses of GCs seems a more promising therapy, but additional studies are still required [214].

GC resistance has been described in various diseases, including leukemia, asthma and COPD [130, 123]. Diverse mechanisms underlying the GC resistance in those diseases have been proposed including activation of MAPK pathway. In PBMCs from patients with asthma, lower ligand affinity of GR was observed. This decrease was also mimicked by challenge with IL-2 and IL-4, which impaired inhibition of pro-inflammatory mediators and induction of IL-10 by GCs. Inhibition of p38 MAPK restored GC sensitivity suggesting that GR phosphorylation by p38 decreases its ligand affinity and causes GC resistance [215]. Another study showed that IL-2 and IL-4 activate p38 MAPK and impair GR S211 phosphorylation and nuclear translocation in PBMCs from healthy subjects [169]. In alveolar macrophages from patients with asthma, impaired induction of MKP-1 (DUSP1) by GCs results in sustained p38 MAPK activation and pro-inflammatory cytokine and chemokine release [126]. In this thesis, we show that MAPK-related GC resistance occurs in lung endothelial cells upon challenge with *Plasmodium berghei* NK65 and IFN-γ.

The effects of GC and the regulation of GC sensitivity have been extensively studied in various immune cells [86]. However, a limited number of studies addressed these issues in endothelial cells, which represent an important target for GCs. Several studies investigated the effects of GC on endothelial cells in inflammatory diseases. For example, signaling through endothelial GR plays a

beneficial role in models of sepsis. Mice with an endothelium-specific deletion of GR show increased mortality, higher levels of TNF, IL-6 and nitric oxide in comparison with control mice after challenge with LPS [216]. GR deletion in HUVECs treated with LPS increases NF-κB levels and IL-6 levels suggesting that endothelial GR plays a crucial role in the regulation of the NF-κB pathway and nitric oxide synthesis. Moreover, mice lacking endothelial GR pre-treated with dexamethasone show increased NF-κB activity compared to wild-type mice after LPS injection [217]. Endothelial GR also plays an important role in the atheroprotective actions of endogenous GCs. Apolipoprotein E (ApoE) KO mice lacking endothelial GR subjected to a high-fat diet developed more severe atherosclerotic lesions and showed increased macrophage recruitment [218].

In this thesis, we performed genome wide analysis of GC action on IFN-γ-induced inflammation in lung endothelial cells. We show that GCs inhibit several clusters of pro-inflammatory mediators and induce a number of protective molecules. To our knowledge, this is the first high-throughput analysis of GC actions on IFN-γ-induced transcriptome in endothelial cells. Moreover, we identify a novel mechanism by which GCs block pro-inflammatory chemokine expression in lung endothelial cells challenged with IFN-γ. We also show that malaria parasites interfere with anti-inflammatory actions of GCs in lung endothelial cells and impair GC-induced MAPK inhibition.

This work offers an interesting explanation of our previous observation in the preclinical model of MA-ARDS, namely that GCs could only block the pulmonary inflammation at extremely high dosages and were even then not able to downregulate the expression of several inflammatory genes in the lungs [8]. As future perspectives, we propose to validate our *in vitro* results in mice and use MAPK inhibitors. MAPK inhibitors could help to reverse GC resistance in patients with asthma [215]. The remaining question in our *in vivo* model of MA-ARDS is whether MAPK inhibitors should be combined with anti-malarial

treatment and/or with GCs. MAPK inhibitors combined with GCs might exert too much immunosuppression. On the other hand, we show that MAPK inhibition blocks the expression of pro-inflammatory chemokines in lung endothelial cells. Therefore, MAPK inhibitors combined with anti-malarial compounds might be a more interesting idea to validate our data *in vivo*. We also aim to validate the data obtained so far in the human *in vitro* model with *P. falciparum*. Moreover, in view of the complex pathogenesis of MA-ARDS, we think that extending our *in vitro* model to other cell types such as macrophages or T cells would provide further interesting insights into how GCs resolve lung inflammation.

In conclusion, this doctoral thesis provides a unique view on the regulation of IFN-γ-induced lung inflammation and identifies *Pb*NK65 as a novel inducer of GC resistance in lung endothelial cells. Throughout different chapters in this work, we confirmed that GC resistance is not caused by failure in GR translocation or phosphorylation. We also show that the STAT1 pathway is not a main target of GCs in our model. In contrast, GCs decreased IFN-γ-induced MAPK signaling and impaired inhibition of MAPK signaling upon challenge with *Pb*NK65 and IFN-γ results in a sustained inflammatory response. Genome-wide analysis of GC actions on lung endothelial cells challenged with IFN-γ further confirmed GC sensitivity of these cells. Our research provides novel insights into the actions of GCs during lung inflammation and also confirms the importance of endothelial cells as a target for GCs. We hope that this work will stimulate further research in different areas including MA-ARDS, ARDS and vascular biology.

Contributions

The thesis manuscript was written by Karolina Zielinska and further edited by Philippe Van den Steen, Karolien De Bosscher, and Ghislain Opdenakker. Karolina Zielinska, Lode De Cauwer, Sofie Knoops, Kristof Van der Molen, Jonathan Thommis, and Alexander Sneyers performed experiments. Karolina Zielinska, Lode De Cauwer, Alexander Sneyers, Karolien De Bosscher, and Philippe Van den Steen analyzed and interpreted the data. Philippe Van den Steen, Karolien De Bosscher, Karolina Zielinska, Ghislain Opdenakker, and J. Brian de Souza conceived the study and designed the experiments.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acronyms

11β-HSD2 11β-hydroxysteroid dehydrogenase 2. 7, 18

11β-HSD1 11β-hydroxysteroid dehydrogenase 1. 7, 18

AF activation function. 7, 8

ALL acute lymphoblastic leukemia. 16

Angptl angiopoietin-like protein. 28

AnxA1 annexin A1. 13, 15

AP-1 activator protein 1. 8, 10, 13, 15, 28, 48, 60

ApoE apolipoprotein E. 61

ARDS acute respiratory distress syndrome. 2, 60, 63

AT1 angiotensin II type 1 receptor. 4

AT2 angiotensin II type 2 receptor. 4

BAG1 BCL2-associated athanogene 1. 17, 19

CBG corticosteroid-binding globulin. 6, 15

CCL CC chemokine ligand. 4, 5, 14, 15, 22, 23, 26, 28, 29, 32, 48, 51, 53, 59

CDK cyclin dependent kinase. 40

COPD chronic obstructive pulmonary disease. 16, 61

COX2 cyclooxygenase 2. 4

CXCL CXC chemokine ligand. 5, 14, 15, 22, 23, 26, 28, 29, 32, 48, 51, 53, 59

DBD DNA binding domain. 7, 8

DC domain cassette. 3

DCs dendritic cells. 12

DUSP1 dual-specificity phosphatase-1. 13, 16, 26, 29, 32, 48, 57, 61

EPCR endothelial protein C receptor. 3

ERK extracellular signal-regulated kinase. 47, 48, 49, 51, 53, 55

FoxP3 transcription factor forkhead box P3. 12

GBP guanylate binding protein. 26, 28

GC glucocorticoid. 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 23, 26, 28, 29, 32, 33, 29, 32, 40, 42, 46, 47, 49, 51, 53, 55, 57, 58, 59, 60, 61, 62, 63

GILZ glucocorticoid-induced leucine zipper. 8, 11, 12, 13, 26, 28, 29, 32, 57

GM-CSF granulocyte-macrophage colony-stimulating factor. 33

GR glucocorticoid receptor. 7, 8, 10, 11, 15, 16, 17, 18, 19, 20, 28, 33, 40, 41, 42, 43, 46, 55, 57, 59, 61, 63

GRE glucocorticoid response element. 7, 8, 10, 28, 33, 57

GRIP1 GR-interacting protein 1. 53

GRO-α growth related oncogene-α. 5

HDAC2 histone deacetylase 2. 10, 16, 17, 33

HPA hypothalamic-pituitary-adrenal. 6

HUVEC human umbilical vein endothelial cell. 17, 19, 46, 55, 57, 61

IFNGR IFN-γ receptor. 47, 49, 59

IRF interferon regulatory factor. 28, 53

I-TAC interferon-inducible T cell α chemoattractant. 5

ICAM-1 intercellular adhesion molecule 1. 4, 5, 14, 15, 22, 26, 32

IFN-γ interferon-γ. 4, 11, 13, 14, 20, 22, 23, 24, 26, 28, 29, 32, 33, 29, 32, 40, 41, 42, 43, 46, 47, 48, 49, 51, 53, 55, 53, 57, 58, 59, 61, 62, 63

IL interleukin. 4, 5, 11, 12, 13, 14, 15, 22, 28, 33, 46, 48, 55, 61

IP-10 interferon-γ-induced protein 10. 5, 22, 23, 26, 29, 32, 48, 51, 53, 59

JAK Janus activated kinase. 47, 48

JNK c-Jun N-terminal kinase. 12, 40, 47, 48, 49, 51, 53, 55, 53, 55, 57, 58, 59

LBD C-terminal ligand-binding domain. 7, 8

LPS lipopolysaccharide. 12, 13, 28, 33, 48, 55, 57, 61

MA-ARDS malaria-associated acute respiratory distress syndrome. 1, 2, 20, 29, 59, 60, 62, 63

MAPK mitogen-activated protein kinase. 4, 12, 13, 20, 28, 40, 47, 48, 49, 51, 53, 55, 57, 59, 61, 62, 63

MCP-1 monocyte chemoattractant protein 1. 4, 5, 14, 15, 22, 23, 26, 29, 32, 51, 53, 59

MHC major histocompatibility complex. 13

MIG monokine induced by IFN-γ. 5, 22, 26, 28, 48

MIP-3α macrophage inflammatory protein-3α. 5

MKK7 mitogen-activated protein kinase kinase 7. 55

MKP-1 map kinase phosphatase-1. 13, 15, 16, 26, 29, 32, 48, 57, 61

MMP-9 matrix metallopeptidase 9. 14, 15, 59

MR mineralocorticoid receptor. 7, 15

MS multiple sclerosis. 12, 14

NF-κB nuclear factor κB. 10, 13, 15, 28, 60, 61

NLS nuclear localization signal. 7, 8

NTD N-terminal transactivation domain. 7, 8

PBMC peripheral blood mononuclear cell. 16, 33, 46, 53, 61

PECAM-1 platelet endothelial cell adhesion molecule 1. 4, 5

PfEMP1 *P. falciparum* erythrocyte membrane protein 1. 3

PK protein kinase. 12

RANTES regulated on activation, normal T cell expressed and secreted. 22, 23, 26, 28, 29, 32, 48, 51, 53, 59

RBC red blood cell. 1, 3, 4, 23, 24, 32, 42, 43, 49, 51, 53, 59

SphK1 sphingosine kinase 1. 8, 15

STAT signal transducer and activator of transcription. 10, 20, 28, 47, 48, 49, 51, 53, 59, 63

TCR T cell receptor. 12

TGF-β transforming growth factor β. 12, 28, 33, 46

TIF2 transcriptional mediators/intermediary factor 2. 53

TIMP tissue inhibitor of metalloproteinases. 14, 15

TLR toll-like receptor. 12, 28, 53

TNF tumor necrosis factor. 4, 5, 6, 12, 22, 32, 33, 46, 48, 55, 57, 61

TNFRSf tumor necrosis factor receptor superfamily member. 28

Tsc22d3 TGF-stimulated clone 22 domain protein-3. 8, 26, 28, 29, 57

TTP tristetraprolin. 13, 15

VCAM-1 vascular cell adhesion molecule 1. 4, 5, 14, 15, 26, 32

VEGF vascular endothelial growth factor. 5

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Karolina Zielińska

Rega Institute KU Leuven
Herestraat 49,3000 Leuven, Belgium
✉ karolina.zielinska@kuleuven.be
k.j.zielinska@gmail.com

Education

2013–2017 **Ph.D. student**.
KU Leuven, Belgium

2012 **M.Sc Biology**.
University of Lodz, Poland

2010 **B.Sc Biology**.
University of Lodz, Poland

2009 **M.Sc Mathematics**.
Lodz University of Technology, Poland

Research Experience

2013–2017 PhD researcher at KU Leuven: Mechanisms of glucocorticoid resistance in malaria associated acute respiratory distress syndrome (MA-ARDS)
Developed in vitro model of MA-ARDS with lung endothelial cells to study GC sensitivity; Investigated mechanism of GC resistance by ELISA, qPCR, immunofluorescence microscopy, siRNA and genome wide analysis of transcription – sample preparation and RNA-Seq data analysis with R and Bioconductor, gained basic experience in FACS

Advanced Training

2017 Introduction to Linux, KU Leuven
How to write a winning grant proposal, VIB

2016 Creating Effective Posters, KU Leuven
Effective Oral Presentations, VIB

2015 RNA-Seq analysis for differential expression, VIB
Basic Bioinformatics Tools and Concepts, VIB

2014 Scientific Writing for Biomedical Sciences, KU Leuven
Biological Data Visualization – the Art of Presenting Data, VIB

Statistical Bioinformatics Using R and Bioconductor, EMBL Heidelberg

Laboratory Animal Sciences, KU Leuven

Publications

K. A. Zielińska, L. Van Moortel, G. Opdenakker, K. De Bosscher, and P. E. Van den Steen, "Endothelial Response to Glucocorticoids in Inflammatory Diseases," *Front Immunol*, vol. 7, no. December, pp. 1–20, 2016.

K. A. Zielińska, L. De Cauwer, S. Knoops, K. Van der Molen, A. Sneyers, B. J. De Souza, J. Thommis, G. Opdenakker, K. De Bosscher, and P. E. Van den Steen, "Plasmodium berghei NK65 in combination with IFN-γ induces endothelial glucocorticoid resistance via sustained activation of p38 and JNK," *Front Immunol*, vol. 8, 2017.

Teaching Experience

2010–2013 **Teaching Assistant**, *Lodz University of Technology, Poland*, Calculus I and II, Differential Equations.

2009–2010 **Teaching Assistant**, *University of Lodz, Poland*, Calculus I.

Thesis Supervision

Supervision of master students.
Styliani Iliaki (2014-2015), Alexander Sneyers (2016-2017), KU Leuven, Belgium

Related Professional Experience

Oct 2012–Jan 2013 **Research Assistant**, *University of Basel, Switzerland*.

Conference Presentations

29th May – 1st June 2017 **2nd Joint Meeting of the European Society for Microcirculation (ESM) and European Vascular Biology Organization (EVBO), Geneva**, *Microvascular Lung Endothelial Cells Become Glucocorticoid Resistant upon Stimulation with Malaria Parasites and Interferon-γ*, Oral presentation.

30th Nov 2016 **Nuclear Receptor Research Network Meeting, Amsterdam**, Malaria parasites in combination with interferon-γ induce glucocorticoid resistance in lung endothelium, Poster presentation.

25th Nov 2016 **Belgian Immunological Society Meeting, Brussels**, Malaria parasites in combination with interferon-γ induce endothelial glucocorticoid resistance in malaria-associated acute respiratory distress syndrome, Poster presentation.

Languages

Polish **Native**
English **Excellent**
Spanish **Good**
German **Basic**

References

PhD supervisor **Prof. Philippe Van den Steen**, *Laboratory of Immunobiology*, Rega Institute for Medical Research-KU Leuven, Herestraat 49 3000 Leuven, Belgium.
philippe.vandensteen@kuleuven.be

Co-supervisor **Prof. Karolien De Bosscher**, *VIB-UGent Center for Medical Biotechnology*, Albert Baertsoenkaai 3, 9000 Gent Belgium.
karolien.debosscher@vib-ugent.be

Co-supervisor **Prof. Ghislain Opdenakker**, *Laboratory of Immunobiology*, Rega Institute for Medical Research-KU Leuven, Herestraat 49 3000 Leuven, Belgium.
ghislain.opdenakker@kuleuven.be

FACULTY OF MEDICINE
DEPT OF MICROBIOLOGY AND IMMUNOLOGY
LABORATORY OF IMMUNOBIOLOGY
Rega Institute, Herestraat 49
3000 Leuven, Belgium
karolina.zielinska@kuleuven.be